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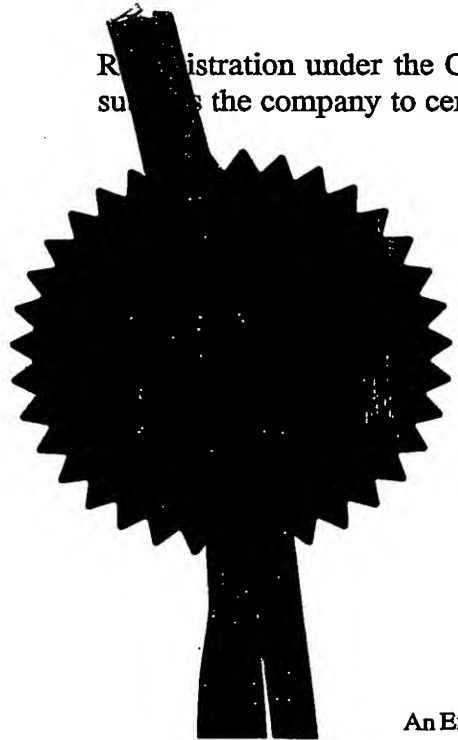
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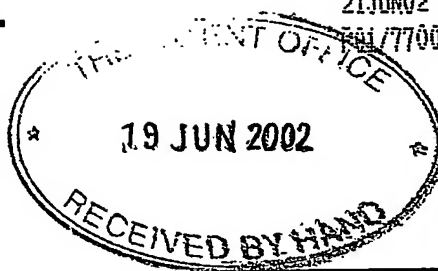
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NORWAY

Patents ADP number (if you know it)

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NORWAY

7946734001

4. Title of the invention

METHOD FOR DETECTING HUMAN PAPILLOMAVIRUS
mRNA

5. Name of your agent (if you have one)

BOULT WADE TENNANT

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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Description 84

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Priority documents

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

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11

I/We request the grant of a patent on the basis of this application.

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METHOD FOR DETECTING HUMAN PAPILLOMAVIRUS mRNA

Field of the invention

5 The present invention relates to *in vitro* methods of screening human subjects in order to assess their risk of developing cervical carcinoma.

Background to the invention

10 Cervical carcinoma is one of the most common malignant diseases world-wide and is one of the leading causes of morbidity and mortality among women (Parkin DM, Pisani P, Ferlay J (1993) Int J Cancer 54: 594-606; Pisani P, Parkin DM, Ferlay J (1993) Int J Cancer 55: 891-903). 15,700 new cases of invasive
15 cervical cancer were predicted in the United States in 1996, and the annual world-wide incidence is estimated to be 450,000 by the World Health Organization (1990). The annual incidence rate differs in different parts of the world, ranging from 7.6 per 100,000 in western
20 Asia to 46.8 per 100,000 in southern Africa (Parkin et al., 1993 *ibid*).

The current conception of cervical carcinoma is that it is a multistage disease, often developing over
25 a period of 10-25 years. Invasive squamous-cell carcinoma of the cervix is represented by penetration through the basal lamina and invading the stroma or epithelial lamina propria. The clinical course of cervical carcinoma shows considerable variation.
30 Prognosis has been related to clinical stage, lymph node involvement, primary tumour mass, histology type, depth of invasion and lymphatic permeation (Delgado G, et al., (1990) Gynecol Oncol 38: 352-357). Some patients with less favourable tumour characteristics
35 have a relatively good outcome, while others suffer a fatal outcome of an initially limited disease. This

shows a clear need for additional markers to further characterise newly diagnosed cervical carcinomas, in order to administer risk-adapted therapy (Ikenberg H, et al., Int. J. Cancer 59:322-6. 1994).

5

The epidemiology of cervical cancer has shown strong association with religious, marital and sexual patterns. Almost 100 case-control studies have examined the relationship between HPV and cervical
~~10 neoplasia and almost all have found positive~~
associations (IARC monographs, 1995). The association is strong, consistent and specific to a limited number of viral types (Munoz N, Bosch FX (1992) HPV and cervical neoplasia: review of case-control and cohort
15 studies. IARC Sci Publ 251-261). Among the most informative studies, strong associations with HPV 16 DNA have been observed with remarkable consistency for invasive cancer and high-grade CIN lesions, ruling out the possibility that this association can be explained
20 by chance, bias or confounding (IARC monographs, 1995). Indirect evidence suggested that HPV DNA detected in cancer cells is a good marker for the role of HPV infection earlier in the carcinogenesis. Dose-response relationship has been reported between
25 increasing viral load and risk of cervical carcinoma (Munoz and Bosch, 1992 *ibid*). In some larger series up to 100% of the tumours were positive for HPV but the existence of virus-negative cervical carcinomas is still debatable (Meijer CJ, et al., (1992) Detection
30 of human papillomavirus in cervical scrapes by the polymerase chain reaction in relation to cytology: possible implications for cervical cancer screening. IARC Sci Publ 271-281; Das BC, et al., (1993) Cancer
72: 147-153).

35

The most frequent HPV types found in squamous-cell cervical carcinomas are HPV 16 (41%-86%)

and 18 (2%-22%). In addition HPV 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 61, 66 and 68 are also found (IARC, monographs, 1995). In the HPV2000 International conference in Barcelona HPV 16, 18, 31 and 45 were defined as high risk, while HPV 33, 35, 39, 51, 52, 56, 58, 59, 68 were defined as intermediate risk (Keerti V. Shah. P71). The 13 high risk plus intermediate risk HPVs are together often referred to as cancer-associated HPV types.

10

A number of studies have explored the potential role of HPV testing in cervical screening (see Cuzick et al. A systematic review of the role of human papillomavirus testing withing a cervical screening programme. Health Technol Assess 3:14. 1999).

15

Reid et al., (Reid R, et al., (1991) Am J Obstet Gynecol 164: 1461-1469) where the first to demonstrate a role for HPV testing in a screening context. This study was carried out on high-risk women from sexually transmitted disease clinics and specialist gynaecologists, and used a sensitive (low stringency) Southern blot hybridisation for HPV detection. A total of 1012 women were enrolled, and cervicography was also considered as a possible adjunct to cytology. Twenty-three CIN II/III lesions were found altogether, but only 12 were detected by cytology (sensitivity 52%, specificity 92%). HPV testing found 16 high-grade lesions.

30

Bauer et al. (Bauer HM, et al., (1991) JAMA 265: 472-477) report an early PCR-based study using MY09/11 primers (Manos M, et al., (1990) Lancet 335: 734) in young women attending for routine smears (college students). They found a positive rate of 46% in 467 women, which was much higher than for dot blot assay (11%).

35

In a study using PCR with GP5/6 primers (Van Den Brule AJ, et al., (1990) J Clin Microbiol 28: 2739-2743) van der Brule et al. (Van Den Brule AJ, et al., (1991) Int J Cancer 48: 404-408) showed a very strong correlation of HPV positivity with cervical neoplasia as assessed by cytology. In older women (aged 35-55 years) with negative cytology the HPV positivity rate was only 3.5%, and this was reduced to 1.5% if only types 16, 18, 31 and 33 were considered, while women with histological carcinoma in situ were all HPV-positive, and 90% had one of the four above types. Women with less severe cytological abnormalities had lower HPV positivity rates in a graded way, showing a clear trend.

Roda Housman et al. (Roda Housman AM, et al., (1994) Int J Cancer 56: 802-806) expanded these observations by looking at a further 1373 women with abnormal smears. This study also confirmed increasing positivity rate with increasing severity of smear results. They also noted that the level of HPV heterogeneity decreased from 22 types for low-grade smears to ten "high-risk" types for high grade smears. This paper did not include any cytologically negative women, nor was cytological disease confirmed histologically.

Cuzick et al. (Cuzick J, et al., (1992) Lancet 340: 112-113; Cuzick J, et al., (1994) Br J Cancer 69: 167-171) were the first to report that HPV testing provided useful information for the triage of cytological abnormalities detected during random screening. In a study of 133 women, referral for colposcopy they found a positive predictive value of 42%, which was similar to that for moderate dyskaryosis. The results were most striking for HPV 16, where 39 of 42 HPV 16 positive women were found to

have high-grade CIN on biopsy. This study pointed out the importance of assessing viral load and only considered high levels of high-risk types as positive.

5 Cox et al. (Cox JT, et al., (1995) Am J Obstet
Gynecol 172: 946-954) demonstrated a role for HPV
testing using the Hybrid Capture™ system (DIGENE
Corporation, Gaithersburg, MD, USA) for triaging women
with borderline smears. This test was performed on
10 217 such women from a college referral service, and a
sensitivity of 93% was found for CINII/III compared
with 73% for repeat cytology. High viral load was
found to further improve performance by reducing false
positives. When 5 RLU was taken as a cut-off, a PPV
15 of approximately 24% was found with no loss of
sensitivity.

 Cuzick et al. (Cuzick J, et al., (1995) Lancet
345: 1533-1536) evaluated HPV testing in a primary
20 screening context in 1985 women attending for routine
screening at a family planning clinic. Sensitivity
using type-specific PCR for the four common HPV types
(75%) exceeded that of cytology (46%), and the PPV for
a positive HPV test (42%) was similar to that for
25 moderate dyskaryosis (43%).

 WO 91/08312 describes methods for determining the
prognosis of individuals infected with HPV which
comprise measuring the level of HPV activity by
30 detecting transcripts of all or a portion of the E6
and/or E7 HPV genes in a sample and comparing the
measurements of HPV activity with a previously
established relationship between activity and risk of
progression to serious cervical dysplasia or
35 carcinoma.

 WO 99/29890 describes methods for the assessment

of HPV infection based on the measurement and analysis of gene expression levels. In particular, WO 99/29890 describes methods which are based on measuring the levels of expression of two or more HPV genes (e.g. HPV E6, E7, L1 and E2) and then comparing the ratio of expression of combinations of these genes to provide an indication of the stage of HPV-based disease in a patient.

10 The present inventors have determined that it is possible to make a clinically useful assessment of HPV-associated disease based only on a simple positive/negative determination of expression of HPV L1 and E6 mRNA transcripts, with no requirement for accurate quantitative measurements of expression levels or for determination of differences in the levels of expression of the two transcripts. This method is technically simple and, in a preferred embodiment, is amenable to automation in a mid-to-high throughput format. Furthermore, on the basis of results obtained using the method of the invention the inventors have defined a novel scheme for classification of patients on the basis of risk of developing cervical carcinoma which is related to disease-relevant molecular changes in the pattern of HPV gene expression and is independent of CIN classification.

30 Therefore, in a first aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or full length E6 mRNA are scored as being at risk of developing cervical carcinoma.

A positive screening result in the method of the invention is indicated by positive expression of L1 mRNA and/or E6 mRNA in cells of the cervix. Positive expression of either one of these mRNAs or both mRNAs is taken as an indication that the subject is "at risk" for development of cervical carcinoma. Women who express E6 mRNA are at high risk of developing cell changes because oncogenic E6 and E7 bind to cell cycle regulatory proteins and act as a switch for cell proliferation. Clear expression of E6 mRNA provides a direct indication of cell changes in the cervix. Expression of L1 mRNA, with or without expression of E6 mRNA is also indicative of the presence of an active HPV.

In the wider context of cervical screening, women identified as positive for L1 and/or E6 mRNA expression may be selected for further investigation, for example using cytology. Thus, at one level the method of the invention may provide a technical simple means of pre-screening a population of women in order to identify HPV-positive subjects who may be selected for further investigation.

In a specific embodiment, the method of the invention may be used to classify subjects into four different classes of risk for developing cervical carcinoma on the basis of positive/negative scoring of expression of L1 and E6 mRNA.

Accordingly, in a further aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of HPV and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of

risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

5 Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 or 68. Those individuals positive for expression of E6 mRNA from at least one of HPV
10 ~~types 16, 18, 31 or 33 are scored as being at higher~~
risk, for example in comparison to individuals negative for these types but positive for expression of E6 mRNA from at least one of HPV types 35, 39, 45, 52, 56, 58, 59, 66 or 68.

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Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 or 68. Those individuals positive
20 for expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33 are scored as being at higher risk, for example in comparison to individuals negative for these types but positive for expression of E6 mRNA from at least one of HPV types 35, 39, 45,
25 52, 56, 58, 59, 66 or 68.

Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA from the cancer-associated HPV types, (e.g. negative for
30 expression of E6 mRNA from HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68).

Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

35

In a preferred embodiment, positive expression is indicated by the presence of more than 50 copies of

the transcript per ml (or total volume of the sample) and negative expression is indicated by the presence of less than 1 copy of the transcript per ml (or total volume of the sample).

5

The above classification is based on molecular events which are relevant to risk of developing cervical carcinoma and is independent of the CIN status of the subjects. Thus, this method of classification may provide an alternative to the use of cytology in the routine screening of women to identify those at potential risk of developing cervical carcinoma. The method may also be used as an adjunct to cytology, for example as a confirmatory test to confirm a risk assessment made on the basis of cytology.

Women positive for expression of high risk E6 mRNA from one of HPV types 16, 18, 31 or 33 but negative for expression of L1 are in the highest level of risk of developing severe cell changes and cell abnormalities. This is due to the fact that a negative result for L1 mRNA expression is directly indicative of integrated HPV, and therefore a higher probability of high and constant expression of E6 and E7. Integration of a virus in the human genome has also a direct impact on the stability of the cells. Integration of HPV also reduces the possibility of regression of cell changes.

30

Women positive for expression of E6 mRNA from one of HPV types 16, 18, 31 or 33 and positive for expression of L1 mRNA have a "high risk" HPV expression and it is still possible that the HPV has been integrated. However, the risk of these women is not classed as high as those who are L1 negative and E6 positive, since there is a reasonable probability

35

that they do not have integrated HPV.

Women negative for expression of E6 mRNA from HPV types 16, 18, 31 or 33 but positive for expression of E6 mRNA from another HPV type, e.g. 35, 39, 45, 52, 56, 58, 59, 66 and 68, are still considered "at risk" and may therefore be placed in risk categories 1 or 2 (as defined above) depending on whether they are positive or negative for expression of L1 mRNA.

10

Women positive for L1 mRNA but negative for E6 mRNA are scored as being at moderate risk. There may be high-risk HPV types in the sample and L1 expression is indicative of lytic activity. There may also be integrated HPV types but only with viruses that are rare. However, detection of lytic activity may show that the cell may soon develop some changes.

15

In the wider context of cervical screening the method of the invention may be used to classify women according to risk of developing cervical carcinoma and therefore provide a basis for decisions concerning treatment and/or further screening. By way of example: women in risk category 1, particularly those who exhibit positive expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33, might be identified as requiring "immediate action", meaning conisation or colposcopy, including a biopsy and histology.

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Women in risk category 2, as defined above, might be scored as requiring immediate attention, meaning colposcopy alone or colposcopy including a biopsy and histology.

35

Women in risk category 3, as defined above, might be scored as requiring immediate re-test, meaning

recall for a further test for HPV expression immediately or after a relatively short interval, e.g. six months.

- 5 Women in risk category 4, as defined above, might be returned to the screening program, to be re-tested for HPV expression at a later date.

10 In a further embodiment the invention provides an *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein
15 subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV.

20 The term "integrated HPV" refers to an HPV genome which is integrated into the human genome.

25 The term "modified episomal HPV genome" is taken to mean an HPV genome which is retained within a cell of the human subject as an episome, i.e. not integrated into the human genome, and which carries a modification as compared to the equivalent wild-type HPV genome, which modification leads to constitutive or persistent expression of transcripts of the E6 and/or E7 genes. The "modification" will typically be
30 a deletion, a multimerisation or concatemerisation of the episome, a re-arrangement of the episome etc affecting the regulation of E6/E7 expression.

35 As aforesaid, the presence of integrated HPV or a modified episomal HPV genome is indicated by a negative result for L1 mRNA expression, together with

a positive result for expression of E6 mRNA in cells of the cervix. Therefore, the ability to predict the presence of integrated HPV or a modified episomal HPV genome in this assay is critically dependent on the ability to score a negative result for L1 mRNA expression. This requires a detection technique which has maximal sensitivity, yet produces minimal false-negative results. In a preferred embodiment this is achieved by using a sensitive amplification and real-

time detection technique to screen for the presence or absence of L1 mRNA. The most preferred technique is real-time NASBA amplification using molecular beacons probes, as described by Leone et al., Nucleic Acids Research., 1998, Vol 26, 2150-2155. Due to the sensitivity of this technique the occurrence of false-negative results is minimised and a result of "negative L1 expression" can be scored with greater confidence.

In a further embodiment, a method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome may be based on screening for expression of E6 mRNA alone. Thus, the invention relates to an *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises screening the subject for expression of mRNA transcripts from the E6 gene of human papillomavirus, wherein subjects positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome.

Moreover, individuals may be sorted into one of two categories of risk for development of cervical carcinoma based on an "on/off" determination of expression of E6 mRNA alone. Therefore, the invention provides an *in vitro* method of screening human

subjects to assess their risk of developing cervical carcinoma, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV and sorting the subject into one of two categories of risk for development of cervical carcinoma based on expression of E6 mRNA, wherein individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified as "high risk" for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV or a modified episomal HPV genome and are therefore classified as "no detectable risk" for development of cervical carcinoma.

Subjects are sorted into one of two categories of risk for development of cervical carcinoma based on an "on/off" determination of expression of E6 mRNA in cells of the cervix. Individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified "high risk" for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV a modified episomal HPV genome and are therefore classified as "no detectable risk" for development of cervical carcinoma.

In the context of cervical screening classification of subjects into the two groups having "high risk" or "no detectable risk" for development of cervical carcinoma provides a basis for decisions concerning treatment and/or further screening. For example subjects in the high risk category may be scored as requiring immediate further analysis, e.g. by histological colposcopy, whilst those in the no

detectable risk category may be referred back to the screening program at three or five year intervals. These methods are particularly useful for assessing risk of developing carcinoma in subjects known to be infected with HPV, e.g. those testing positive for HPV DNA, or subjects who have previously manifested a cervical abnormality via cytology or pap smear. Subjects placed in the "no detectable risk" category on the basis of E6 mRNA expression may have HPV DNA

present but the negative result for E6 expression indicates that HPV is unrelated to oncogene activity at the time of testing.

The presence of integrated HPV or a modified episomal HPV genome, as indicated by a positive result for E6 mRNA expression, is itself indicative that the subject has abnormal cell changes in the cervix. Therefore, the invention also relates to an *in vitro* method of identifying human subjects having abnormal cell changes in the cervix, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV, wherein individuals positive for expression of E6 mRNA are identified as having abnormal cell changes in the cervix.

The term "abnormal cell changes in the cervix" encompasses cell changes which are characteristic of more severe disease than low-grade cervical lesions or low squamous intraepithelial lesions, includes cell changes which are characteristic of disease of equal or greater severity than high-grade CIN (defined as a neoplastic expansion of transformed cells), CIN (cervical intraepithelial neoplasia) III, or high squamous intraepithelial neoplasia (HSIL), including lesions with multiploid DNA profile and "malignant" CIN lesions with increased mean DNA-index values, high percentage of DNA-aneuploidy and 2.5c Exceeding Rates

(Hanselaar et al., 1992, Anal Cell Pathol., 4:315-324; Rihet et al., 1996, J. Clin Pathol 49:892-896; and McDermott et al., 1997, Br. J. Obstet Gynaecol. 104:623-625).

5

Cervical Intraepithelial Neoplasia (abbreviated "CIN"), also called Cervical Dysplasia, is a cervical condition caused Human Papilloma Virus. CIN is classified as I, II or III depending on its severity. It is considered a pre-cancerous abnormality, but not an actual cancer. The mildest form, CIN I, usually goes away on its own, although rarely it can progress to cancer. The more severe forms, CIN II and CIN III, most often stay the same or get worse with time. They can become a cancer, but almost never do if treated adequately.

HPV has been identified as a causative agent in development of cellular changes in the cervix, which may lead to the development of cervical carcinoma. These cellular changes are associated with constitutive or persistent expression of E6/E7 proteins from the HPV viral genome. Thus, it is possible to conclude that subjects in which expression of E6 mRNA can be detected, particularly those subjects who exhibit persistent E6 expression when assessed over a period of time, already manifest cellular changes in the cervix. These changes may have taken place in only a very few cells of the cervix, and may not be detectable by conventional cytology. Nevertheless, with the use of sensitive, specific and accurate methods for detection of E6 mRNA it is possible to identify those subjects who already exhibit cellular changes in the cervix at a much earlier stage than would be possible using conventional cytological screening. This will allow earlier intervention with treatments aimed at

preventing the development of cervical carcinoma.

As a result of HPV integration into the human genome or as a result of the "modification" in a modified episomal HPV genome, normal control of the viral E6/E7 oncogene transcription is lost (Durst et al., 1985, J Gen Virol, 66(Pt 7): 1515-1522; Pater and Pater, 1985 Virology 145:313-318; Schwarz et al., 1985, Nature 314: 111-114; Park et al., 1997, *ibid*).

10 In contrast, in premalignant lesions and HPV-infected normal epithelium papillomaviruses predominate in "unmodified" episomal forms, hence oncogene (E6/E7) transcription may be absent or efficiently down-regulated (Johnson et al., 1990, J Gen Virol, 71(Pt 7): 1473-1479; Falcinelli et al., 1993, J Med Virol, 40: 261-265). Integration of human papillomavirus type 16 DNA into the human genome is observed to lead to a more unstable cell activity/genome, and increased stability of E6 and E7 mRNAs (Jeon and Lambert, 1995, Proc Natl Acad Sci USA 92: 1654-1658). Thus HPV integration, typically found in cervical cancers but only infrequently found in CIN lesions (Carmody et al., 1996, Mol Cell Probes, 10: 107-116), appears to be an important event in cervical carcinogenesis.

The present methods detect E6/E7 viral mRNA expression in the cervix instead of DNA. E6/E7 viral expression in cervical cells is a much more accurate assessment of the risk of developing cancer than simply showing that the HPV virus is present. Furthermore, the detection of HPV oncogene transcripts may be a more sensitive indicator of the direct involvement of viral oncogenes in carcinogenesis (Rose et al., 1994, Gynecol Oncol, 52: 212-217; Rose et al., 1995, Gynecol Oncol, 56: 239-244). Detection of E6/E7

transcripts by amplification and detection is a useful
diagnostic tool for risk evaluations regarding the
development of CIN and its progression to cervical
cancer, especially in high-risk HPV type-infected
5 patients with ASCUS and CIN I (Sotlar et al., 1998,
Gynecol Oncol, 69: 114-121; Selinka et al., 1998, Lab
Invest, 78: 9-18).

10 The expression of E6/E7 transcripts of HPV-16/18
is uniformly correlated with the physical status of
HPV DNAs (Park et al., 1997, Gynecol Oncol, Vol:65(1),
121-9). In most cervical carcinoma cells the E6 and
E7 genes of specific human papillomaviruses are
transcribed from viral sequences integrated into host
15 cell chromosomes (von Kleben Doeberitz et al., 1991,
Proc Natl Acad Sci U S A. Vol:88(4), 1411-5). Viral
load and integration has been evaluated in a large
series of CIN lesions (Pietsaro et al., 2002, J Clin
Microbiol, Vol:40(3), 886-91). Only one sample
20 contained exclusively episomal HPV16 DNA, and this
lesion regressed spontaneously. Seventeen of 37
invasive cervical carcinoma samples were identified
previously as containing the completely integrated
HPV16 genome by using PCR covering the entire E1/E2
25 gene, and this was confirmed by rliPCR in 16 cases.
One case, however, showed a low level of episomal
deoxyribonucleic acid in addition to the predominant
integrated form. Of the remaining 20 carcinoma
samples showing episomal forms in the previous
30 analysis, 14 were found to contain integrated forms
using rliPCR, and four contained multimeric (modified)
episomal forms. Thus, in total, 31 of 37 of the
carcinomas (84%) showed integrated HPV16 genome, while
absence of integration could not be detected.
35 (Kalantari et al., 2001, Diagn Mol Pathol, Vol:10(1),
46-54).

There have been virtually no observations that cervical carcinoma cells exist without integrated HPV or modified episomal HPV DNA (Kalantari et al. 2001; Pietsaro et al., 2002, *ibid*). It has further been shown that E6 and E7 may only be transcribed from integrated or modified episomal HPV DNA (von Kleben Doeberitz et al., 1991, *ibid*). Therefore, the inventors surmise that detection of E6/E7 expression provides a direct indication of integrated HPV or

modified episomal HPV and high oncogene activity, and conclude that in a clinical context detection of E6 (E6/E7) expression alone is sufficient to identify subjects at "high risk" of developing cervical carcinoma. In other words, if E6/E7 mRNA expression can be detected in a cervical sample, this is directly indicative of cellular abnormalities in the cervix and there is a very high risk of development of cervical carcinoma due to persistent HPV oncogene activity. Therefore, detection of E6/E7 mRNA in a human subject indicates that the subject has a very high risk of developing cervical carcinoma and should undergo immediate further screening, e.g. by colposcopy.

If HPV E6/E7 mRNA expression is not detected, the subject may still have an HPV infection. However due to absence of integration and oncogene activity, it may regress spontaneously (as observed by Pietsaro et al., 2002, *ibid*).

In a clinical context the performance of methods which rely on screening for expression of E6 mRNA alone is critically dependent on the ability to score a negative result for E6 mRNA expression with confidence. This again requires a detection technique which has maximal sensitivity, yet produces minimal false-negative results. In a preferred embodiment

this is achieved by using a sensitive amplification and real-time detection technique to screen for the presence or absence of E6 mRNA. The most preferred technique is real-time NASBA amplification using
5 molecular beacons probes, as described by Leone et al., Nucleic Acids Research., 1998, Vol 26, 2150-2155. Due to the sensitivity of this technique the occurrence of false-negative results is minimised and a result of "negative E6 expression" can be scored
10 with greater confidence. This is extremely important if the assays are to be used in the context of a clinical screening program.

In the methods based on detection of E6 mRNA
15 alone it is preferred to detect at least types HPV 16, 18, 31, 33 and 45, and in a preferred embodiment the assay may detect only these HPV types. DNA from HPV types 16, 18, 31 and 33 has been detected in more than 87% of cervical carcinoma samples (Karlsen et al.,
20 1996, J Clin Microbiol, 34:2095-2100). Other studies have shown that E6 and E7 are almost invariably retained in cervical cancers, as their expression is likely to be necessary for conversion to and
25 maintenance of the malignant state (Choo et al., 1987, J Med Virol 21:101-107; Durst et al., 1995, Cancer Genet Cytogenet, 85: 105-112). In contrast to HPV detection systems which are based on detection of the undamaged genome or the L1 gene sequence, detection of HPV mRNA expressed from the E6/E7 area may detect more
30 than 90% of the patients directly related to a risk of developing cervical carcinoma.

In the clinic, methods based on detection of E6 mRNA are preferred for use in post-screening, i.e.
35 further analysis of individuals having a previous diagnosis of ASCUS, CIN 1 or Condyloma. The method may be used to select those with a high risk of

developing cervical carcinoma from amongst the group of individuals having a previous diagnosis of ASCUS, CIN 1 or Condyloma. ASCUS, Condyloma and CIN I may be defined as more or less the same diagnosis due to very low reproducibility between different cytologists and different cytological departments. Östör (Int J. Gyn Path. 12:186-192. 1993) found that only around 1% of the CIN 1 cases may progress to cervical carcinoma. Thus, there is a genuine need for an efficient method

10 of identifying the subset of individuals with ASCUS, Condyloma or CIN I who are at substantial risk of developing cervical carcinoma. One of HPV types 16, 18, 31 or 33 was detected in 87% of the cervical carcinoma cases study by Karlsen et al., 1996. By
15 inclusion of HPV 45, nearly 90% of the cervical carcinoma samples are found to be related to these five HPV types. Therefore, calculated from the data provided by Östör (Int J. Gyn Path. 12:186-192. 1993) more than 99.9% are detected cases with ASCUS, CIN I
20 or condyloma are missed by our HPV-Proofer kit.

In the methods of the invention "positive expression" of an mRNA is taken to mean expression above background. There is no absolute requirement
25 for accurate quantitative determination of the level of mRNA expression or for accurate determination of the relative levels of expression of L1 and E6 mRNA.

In certain embodiments, the methods of the
30 invention may comprise a quantitative determination of levels of mRNA expression. In a preferred embodiment in order to provide a clear distinction between "positive expression" and "negative expression" a determination of "positive expression" may require the
35 presence of more than 50 copies of the relevant mRNA (per ml of sample or per total volume of sample), whereas a determination of "negative expression" may

require the presence of less than 1 copy of the relevant mRNA (per ml of sample or per total volume of sample).

5 The methods of the invention will preferably involve screening for E6 mRNA using a technique which is able to detect specifically E6 mRNA from cancer-associated HPV types, more preferably "high risk" cancer-associated HPV types. In the most preferred
10 embodiment the methods involve screening for E6 mRNA using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31 and 33, and preferably also 45. Most preferably, the method will specifically detect
15 expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, and preferably also 45, and most preferably all five types. However, women positive for positive for expression of E6 from other types than 16, 18, 31, 33 and 45, e.g. 35, 39, 45, 52, 56, 58, 59, 66 and 68 may still be "at risk" of developing
20 cervical carcinoma. Thus, the method may encompass screening for expression of E6 mRNA from one or more of these HPV types, most preferably in addition to screening for E6 mRNA from HPV types 16, 18, 31, 33 and 45. Certain HPV types exhibit a marked
25 geographical/population distribution. Therefore, it may be appropriate to include primers specific for an HPV type known to be prevalent in the population/geographical area under test, for example in addition to screening for HPV types 16, 18, 31, 33
30 and 45.

 For the avoidance of doubt, unless otherwise stated the term "E6 mRNA" as used herein encompasses all naturally occurring mRNA transcripts which contain
35 all or part of the E6 open reading frame, including naturally occurring splice variants, and therefore includes transcripts which additionally contain all or

part of the E7 open reading frame (and indeed further open reading frames). The terms "E6/E7 mRNA", "E6/E7 transcripts" etc are used interchangeably with the terms "E6 mRNA", "E6 transcripts" and also encompass naturally occurring mRNA transcripts which contain all or part of the E6 open reading frame, including naturally occurring splice variants, and transcripts which contain all or part of the E7 open reading frame. The term "oncogene expression", unless

otherwise stated, also refers to naturally occurring mRNA transcripts which contain all or part of the E6 open reading frame, including naturally occurring splice variants, and transcripts which contain all or part of the E7 open reading frame.

Four E6/E7 mRNA species have so far been described in cells infected with HPV 16, namely an unspliced E6 transcript and three spliced transcripts denoted E6*I, E6*II and E6*III (Smotkin D, et al., J Virol. 1989 Mar 63(3):1441-7; Smotkin D, Wettstein FO. Proc Natl Acad Sci USA. 1986 Jul 83(13):4680-4; Doorbar J. et al., Virology. 1990 Sep 178(1):254-62; Cornelissen MT, et al. J Gen Virol. 1990 May 71(Pt 5):1243-6; Johnson MA, et al. J Gen Virol. 1990 Jul 71(Pt 7):1473-9; Schneider-Maunoury S, et al. J Virol. 1987 Oct 61(10):3295-8; Sherman L, et al. Int J Cancer. 1992 Feb 50(3):356-64). All four transcripts are transcribed from a single promoter (p97) located just upstream of the second ATG of the E6 ORF.

In one embodiment the methods may comprise screening for E6 transcripts which contain all or part of the E7 open reading frame, This may be accomplished, for example, using primers or probes specific for the E7 coding region.

In a further embodiment, the methods may comprise

screening for the presence of "full length" E6 transcripts. In the case of HPV 16 the term "full length E6 transcripts" refers to transcripts which contain all of the region from nucleotide (nt) 97 to
5 nt 880 in the E6 ORF, inclusive of nt 97 and 880. Nucleotide positions are numbered according to standard HPV nomenclature (see Human Papillomavirus Compendium OnLine, available via the internet or in
10 paper form from HV Database, Mail Stop K710, Los Alamos National Laboratory, Los Alamos, NM 87545, USA). Specific detection of full length transcripts may be accomplished, for example, using primers or
15 probes which are specific for the region which is present only in full length E6 transcripts, not in splice variants. Different HPV types exhibit different patterns of E6/E7 mRNA expression. Transcript maps for various HPV types, including HPV types 16 and 31, which may be used to assist in the design of probes or primers for detection of E6/E7
20 transcripts are publicly available via the Human Papillomavirus Compendium (as above).

E6 oligonucleotide primers are described herein which are suitable for use in amplification of regions
25 of the E6 mRNA from various HPV types by NASBA or PCR.

In a preferred embodiment methods which involve screening for L1 mRNA expression may comprise screening for L1 mRNA expression using a technique
30 which is able to detect L1 mRNA from substantially all known HPV types or at least the major cancer-associated HPV types (e.g. preferably all of HPV types 16, 18, 31 and 33) . L1 primers and probes are described herein which are capable of detecting L1
35 mRNA from HPV types 6, 11, 16, 18, 31, 33, 35 and 51 in cervical samples.

Detection of L1 transcripts can be said to detect HPV "virulence", meaning the presence of HPV lytic activity. Detection of E6/E7 transcripts can be said to detect HPV "pathogenesis" since expression of these
5 mRNAs is indicative of molecular events associated with risk of developing carcinoma.

In a study of 4589 women it was possible to detect all except one case of CIN III lesions or
10 cancer using a method based on screening for expression of E6 and L1 mRNA (see accompanying Examples).

In further embodiments, the above-described
15 methods of the invention may comprise screening for expression of mRNA transcripts from the human p16^{ink4a} gene, in addition to screening for expression of HPV L1 and/or E6 transcripts.

20 A positive result for expression of p16^{ink4a} mRNA is taken as a further indication of risk of developing cervical carcinoma.

P16^{ink4a}, and the related family members, may
25 function to regulate the phosphorylation and the growth suppressive activity of the retinoblastoma gene product (RB). In support of this, it has been found that there is an inverse relationship between the expression of p16^{ink4a} protein and the presence of
30 normal RB in selected cancer cell lines; p16^{ink4a} protein is detectable when RB is mutant, deleted, or inactivated, and it is markedly reduced or absent in cell lines that contain a normal RB. Kheif et al. (Kheif SN et al., Proc. Natl. Acad. Sci. USA
35 93:4350-4354. 1996), found that p16^{ink4a} protein is expressed in human cervical carcinoma cells that contain either a mutant RB or a wild-type RB that is

functionally inactivated by E7. They also show that the inactivation of RB correlates with an upregulation of p16^{ink4a} confirming a feedback loop involving p16^{ink4a} and RB. Milde-Langosch et al. (Milde-Langosch K, et al., (2001) Virchows Arch 439: 55-61) found that there were significant correlations between strong p16 expression and HPV16/18 infection and between strong p16 expression and HPV 16/18 E6/E7 oncogene expression. Klaes et al., (Klaes R, et al., (2001) Int J Cancer 92: 276-284) observed a strong over expression of the p16^{ink4a} gene product in 150 of 152 high-grade dysplastic cervical lesions (CIN II to invasive cancer), whereas normal cervical epithelium or inflammatory or metaplastic lesions were not stained with the p16^{ink4a} specific monoclonal antibody E6H4. All CIN I scored lesions associated with LR-HPV types displayed no or only focal or sporadic reactivity, whereas all but two CIN I scored lesions associated with HR-HPV types showed strong and diffuse staining for p16^{ink4a}.

The disclosed screening methods may be carried out on a preparation of nucleic acid isolated from a clinical sample or biopsy containing cervical cells taken from the subject under test. Suitable samples which may be used as a source of nucleic acid include (but not exclusively) cervical swabs, cervical biopsies, cervical scrapings, skin biopsies/warts, also paraffin embedded tissues, and formalin or methanol fixed cells.

The preparation of nucleic acid to be screened using the disclosed method must include mRNA, however it need not be a preparation of purified poly A+ mRNA and preparations of total RNA or crude preparations of total nucleic acid containing both RNA and genomic DNA, or even crude cell lysates are also suitable as

starting material for a NASBA reaction. Essentially any technique known in the art for the isolation of a preparation of nucleic acid including mRNA may be used to isolate nucleic acid from a test sample. A
5 preferred technique is the "Boom" isolation method described in US-A-5,234,809 and EP-B-0389,063. This method, which can be used to isolate a nucleic acid preparation containing both RNA and DNA, is based on the nucleic acid binding properties of silicon dioxide
10 particles in the presence of the chaotropic agent guanidine thiocyanate (GuSCN).

The methods of the invention are based on assessment of active transcription of the HPV genome
15 in cervical cells. The methods are not limited with respect to the precise technique used to detect mRNA expression. Many techniques for detection of specific mRNA sequences are known in the art and may be used in accordance with the invention. For example, specific
20 mRNAs may be detected by hybridisation, amplification or sequencing techniques.

It is most preferred to detect mRNA expression by means of an amplification technique, most preferably
25 an isothermal amplification such as NASBA, transcription-mediated amplification, signal-mediated amplification of RNA technology, isothermal solution phase amplification, etc. All of these methods are well known in the art. More preferably mRNA expression
30 is detected by an isothermal amplification in combination with real-time detection of the amplification product. The most preferred combination is amplification by NASBA, coupled with real-time detection of the amplification product using molecular
35 beacons technology, as described by Leone et al., Nucleic Acids Research, 1998, Vol 26, 2150-2155.

Methods for the detection of HPV in a test sample using the NASBA technique will generally comprise the following steps:

5 (a) assembling a reaction medium comprising
suitable primer-pairs, an RNA directed DNA polymerase,
a ribonuclease that hydrolyses the RNA strand of an
RNA-DNA hybrid without hydrolysing single or double
stranded RNA or DNA, an RNA polymerase that recognises
said promoter, and ribonucleoside and
10 deoxyribonucleoside triphosphates;

(b) incubating the reaction medium with a
preparation of nucleic acid isolated from a test
sample suspected of containing HPV under reaction
conditions which permit a NASBA amplification
15 reaction; and

(c) detecting and/or quantitatively measuring
any HPV-specific product of the NASBA amplification
reaction.

20 Detection of the specific product(s) of the NASBA
reaction (i.e. sense and/or antisense copies of the
target RNA) may be carried out in a number of
different ways. In one approach the NASBA product(s)
may be detected with the use of an HPV-specific
25 hybridisation probe capable of specifically annealing
to the NASBA product. The hybridisation probe may be
attached to a revealing label, for example a
fluorescent, luminescent, radioactive or
chemiluminescent compound or an enzyme label or any
30 other type of label known to those of ordinary skill
in the art. The precise nature of the label is not
critical, but it should be capable of producing a
signal detectable by external means, either by itself
or in conjunction with one or more additional
35 substances (e.g. the substrate for an enzyme).

A preferred detection method is so-called "real-

time NASBA" which allows continuous monitoring of the formation of the product of the NASBA reaction over the course of the reaction. In a preferred embodiment this may be achieved using a "molecular beacons" probe comprising an HPV-specific sequence capable of annealing to the NASBA product, a stem-duplex forming oligonucleotide sequence and a pair of fluorescer/quencher moieties, as known in the art and described herein. If the molecular beacons probe is

10 added to the reaction mixture prior to amplification it may be possible to monitor the formation of the NASBA product in real-time (Leone et al., Nucleic Acids Research, 1998, Vol 26, 2150-2155). Reagent kits and instrumentation for performing real-time
15 NASBA detection are available commercially (e.g. NucliSens™ EasyQ system, from Organon Teknika).

In a further approach, the molecular beacons technology may be incorporated into the primer 2
20 oligonucleotide allowing real-time monitoring of the NASBA reaction without the need for a separate hybridisation probe.

In a still further approach the products of the
25 NASBA reaction may be monitored using a generic labelled detection probe which hybridises to a nucleotide sequence in the 5' terminus of the primer 2 oligonucleotide. This is equivalent to the "NucliSens™" detection system supplied by Organon
30 Teknika. In this system specificity for NASBA products derived from the target HPV mRNA may be conferred by using HPV-specific capture probes comprising probe oligonucleotides as described herein attached to a solid support such as a magnetic
35 microbead. Most preferably the generic labelled detection probe is the ECL™ detection probe supplied by Organon Teknika. NASBA amplicons are hybridized to

the HPV-specific capture probes and the generic ECL probe (via a complementary sequence on primer 2). Following hybridization the bead/amplicon/ECL probe complexes may be captured at the magnet electrode of an automatic ECL reader (e.g. the NucliSens™ reader supplied by Organon Teknika). Subsequently, a voltage pulse triggers the ECL™ reaction.

The detection of HPV mRNA is also of clinical relevance in cancers other than cervical carcinoma including, for example, head and neck carcinoma, oral and tongue carcinoma, skin carcinoma, anal and vaginal carcinoma. Detection of HPV mRNA may also be very useful in the diagnosis of micrometastases in lymph nodes in the lower part of the body. Hence, the invention also contemplates screens for susceptibility to the above-listed cancers based on screening for expression of HPV L1 and E6 transcripts.

In accordance with a further aspect of the invention there is provided a kit for use in the detection of transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31 and 33, and preferably also HPV 45, and one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31 and 33, and preferably also HPV 45.

"Primer-pair" taken to mean are pair of primers which may be used in combination to amplify a specific region of the L1 or E6 mRNA using any known nucleic acid technique. In preferred embodiments the primer-pairs included in the kit will be suitable for use in NASBA amplification or similar isothermal amplification techniques.

The individual primers making up each primer-pair included in the kit may be supplied separately (e.g. a separate container of each primer) or, more preferably, may be supplied mixed in a single container. Combinations of two or more primer-pairs may be supplied ready-mixed in a single container within the kit. It may be convenient to supply two or more primer-pairs in a single container where the two or more amplification reactions are to be

10 "multiplexed", meaning performed simultaneously in a single reaction vessel.

15 The primer-pair(s) suitable for use in amplification of a region of E6 transcripts should enable amplification a region of E6 mRNA from at least the major cancer-associated HPV types 16, 18, 31 and 33, and preferably also HPV 45. There are several different ways in which this can be achieved.

20 In one embodiment, the kit may contain separate primer-pairs specific for each of HPV types 16, 18, 31 and 33, and preferably also HPV 45. These primer-pairs may be supplied within the kit in separate containers, or they may be supplied as mixtures of two or more primer-pairs in a single container, for example to enable multiplexing of the amplification reactions.

30 In a further embodiment, the kit may contain a single primer-pair capable of amplifying a region of the E6 gene from HPV types 16, 18, 31 and 33, and preferably also HPV 45, which thus enables amplification of all four (preferably five) types in a single amplification reaction. This could, for example, be achieved with the use of a pair of degenerate primers or by selection of a region of the E6 mRNA which is highly conserved across HPV types.

The E6 primer-pair may correspond to any region of the E6 mRNA, and may enable amplification of all or part of the E6 open reading frame and/or the E7 open reading frame.

5

The kit may further include primer-pairs suitable for use in amplification of E6 mRNA from HPV types other than types 16, 18, 31 and 33, and preferably also HPV 45. For example, the kit may be supplemented with E6 primers for detection of an HPV type which is endemic in a particular geographical area or population.

15 The primer-pair(s) suitable for use in amplification of a region of L1 transcripts should be capable of amplifying a region of L1 mRNA from at least the major cancer-associated HPV types 16, 18, 31 and 33, and preferably also HPV 45, and will preferably be suitable for use in amplification of a region of L1 mRNAs from substantially all known HPV types. With the use of such primers it is possible to test for active transcription of L1 mRNA from multiple HPV types in a single amplification reaction.

25 It is possible to design primers capable of detecting L1 transcripts from multiple HPV types by selecting regions of the L1 transcript which are highly conserved.

30 In a further approach, specificity for multiple HPV types may be achieved with the use of degenerate oligonucleotide primers or complex mixtures of polynucleotides which exhibit minor sequence variations, preferably corresponding to sites of sequence variation between HPV genotypes. The rationale behind the use of such degenerate primers or mixtures is that the mixture may contain at least one

35

primer-pair capable of detecting each HPV type.

5 In a still further approach specificity for multiple HPV types may be achieved by incorporating into the primers one or more inosine nucleotides, preferably at sites of sequence variation between HPV genotypes.

10 The E6 and L1 primer-pairs may be supplied in separate containers within the kit, or the L1 primer-pair(s) may be supplied as a mixture with one or more E6 primer-pairs in a single container.

15 The kits may further comprise one or more probes suitable for use in detection of the products of amplification reactions carried out using the primer-pairs included within the kit. The probe(s) may be supplied as a separate reagent within the kit. Alternatively, the probe(s) may be supplied as a
20 mixture with one or more primer-pairs.

25 The primers and probes included in the kit are preferably single stranded DNA molecules. Non-natural synthetic polynucleotides which retain the ability to base-pair with a complementary nucleic acid molecule may also be used, including synthetic oligonucleotides which incorporate modified bases and synthetic oligonucleotides wherein the links between individual
30 nucleosides include bonds other than phosphodiester bonds. The primers and probes may be produced according to techniques well known in the art, such as by chemical synthesis using standard apparatus and protocols for oligonucleotide synthesis.

35 The primers and probes will typically be isolated single-stranded polynucleotides of no more than 100 bases in length, more typically less than 55 bases in

length. For the avoidance of doubt it is hereby stated that the terms "primer" and "probe" exclude naturally occurring full-length HPV genomes.

5 Several general types of oligonucleotide primers and probes incorporating HPV-specific sequences may be included in the kit. Typically, such primers and probes may comprise additional, non-HPV sequences, for example sequences which are required for an
10 amplification reaction or which facilitate detection of the products of the amplification reaction.

 The first type of primers are primer 1 oligonucleotides (also referred to herein as NASBA P1
15 primers), which are oligonucleotides of generally approximately 50 bases in length, containing an average of about 20 bases at the 3' end that are complementary to a region of the target mRNA. Oligonucleotides suitable for use as NASBA P1 primers
20 are denoted "P1/PCR" in Table 1. P1 primer oligonucleotides have the general structure X_1 -SEQ, wherein SEQ represents an HPV-specific sequence and X_1 is a sequence comprising a promoter that is recognized by a specific RNA polymerase. Bacteriophage
25 promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the oligonucleotides of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. In a preferred
30 embodiment, sequence " X_1 " may comprise the sequence AATTCTAATACGACTCACTATAGGG or the sequence AATTCTAATACGACTCACTATAGGGAGAAGG. These sequences contains a T7 promoter, including the transcription initiation site for T7 RNA polymerase.
35 The HPV-specific sequences in the primers denoted in Table 1 as "P1/PCR" may also be adapted for use in standard PCR primers. When these sequences are used

as the basis of NASBA P1 primers they have the general structure X_1 -SEQ, as defined above. The promoter sequence X_1 is essential in a NASBA P1 primer. However, when the same sequences are used as the basis of standard PCR primers it is not necessary to include X_1 .

A second type of primers are NASBA primer 2 oligonucleotides (also referred to herein as NASBA P2

primers) which generally comprise a sequence of approximately 20 bases substantially identical to a region of the target mRNA. The oligonucleotide sequences denoted in Table 1 as "P2/PCR" are suitable for use in both NASBA P2 primers and standard PCR primers.

Oligonucleotides intended for use as NASBA P2 primers may, in a particular but non-limiting embodiment, further comprise a sequence of nucleotides at the 5' end which is unrelated to the target mRNA but which is capable of hybridising to a generic detection probe. The detection probe will preferably be labelled, for example with a fluorescent, luminescent or enzymatic label. In one embodiment the detection probe is labelled with a label that permits detection using ECL™ technology, although it will be appreciated that the invention is in no way limited to this particular method of detection. In a preferred embodiment the 5' end of the primer 2 oligonucleotides may comprise the sequence GATGCAAGGTCGCATATGAG. This sequence is capable of hybridising to a generic ECL™ probe commercially available from Organon Teknika having the following structure:

$\text{Ru}(\text{bpy})_3^{2+}$ -GAT GCA AGG TCG CAT ATG AG-3'

In a different embodiment the primer 2

oligonucleotide may incorporate "molecular beacons" technology, which is known in the art and described, for example, in WO 95/13399 by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996, to allow for
5 real-time monitoring of the NASBA reaction.

Target-specific probe oligonucleotides may also be included within the kit. Probe oligonucleotides generally comprise a sequence of approximately 20-25
10 bases substantially identical to a region of the target mRNA, or the complement thereof. Example HPV-specific oligonucleotide sequences which are suitable for use as probes are denoted "PO" in Table 1. The probe oligonucleotides may be used as target-specific
15 hybridisation probes for detection of the products of a NASBA or PCR reaction. In this connection the probe oligonucleotides may be coupled to a solid support, such as paramagnetic beads, to form a capture probe (see below). In a preferred embodiment the 5' end of
20 the probe oligonucleotide may be labelled with biotin. The addition of a biotin label facilitates attachment of the probe to a solid support via a biotin/streptavidin or biotin/avidin linkage.

25 Target-specific probes enabling real-time detection of amplification products may incorporate "molecular beacons" technology which is known in the art and described, for example, by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996 and in WO
30 95/13399. Example HPV-specific oligonucleotide sequences suitable for use as molecular beacons probes are denoted "MB" in Table 1.

The term "molecular beacons probes" as used
35 herein is taken to mean molecules having the structure:

X_2 -arm₁-target-arm₂-X₃

wherein "target" represents a target-specific sequence of nucleotides, "X₂" and "X₃" represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and "arm₁" and "arm₂" represent complementary sequences capable of forming a stem duplex.

Preferred combinations of "arm₁" and "arm₂" sequences are as follows, however these are intended to be illustrative rather than limiting to the invention:

cgcatg-SEQ-catgcg
ccagct-SEQ-agctgg
cacgc-SEQ-gcgtg
cgatcg-SEQ-cgatcg
ccgtcg-SEQ-cgacgg
cggacc-SEQ-ggtccg
ccgaagg-SEQ-ccttcgg
cacgtcg-SEQ-cgacgtg
cgcagc-SEQ-gctgcg
ccaagc-SEQ-gcttgg
ccaagcg-SEQ-cgcttgg
cccagc-SEQ-gctggg
ccaaagc-SEQ-gctttgg
cctgc-SEQ-gcagg
ccaccc-SEQ-gggtgg
ccaagcc-SEQ-ggcttgg
ccagcg-SEQ-cgctgg
cgcatg-SEQ-catgcg

The use of molecular beacons technology allows for real-time monitoring of amplification reactions,

for example NASBA amplification (see Leone et al.,
Nucleic Acids Research., 1998, vol: 26, pp 2150-2155).
The molecular beacons probes generally include
5 complementary sequences flanking the HPV-specific
sequence, represented herein by the notation arm₁ and
arm₂, which are capable of hybridising to each other
form a stem duplex structure. The precise sequences
of arm₁ and arm₂ are not material to the invention,
except for the requirement that these sequences must
10 be capable of forming a stem duplex when the probe is
not bound to a target HPV sequence.

Molecular beacons probes also include a
fluorescent moiety and a quencher moiety, the
15 fluorescent and the quencher moieties being
represented herein by the notation X₂ and X₃. As will
be appreciated by the skilled reader, the fluorescer
and quencher moieties are selected such that the
quencher moiety is capable of substantially or
20 completely quenching the fluorescence from the
fluorescent moiety when the two moieties are in close
proximity, e.g. when the probe is in the hairpin
"closed" conformation in the absence of the target
sequence. Upon binding to the target sequence, the
25 fluorescent and quencher moieties are held apart such
that the fluorescence of the fluorescent moiety is no
longer quenched.

Many examples of suitable pairs of
30 quencher/fluorescer moieties which may be used in
accordance with the invention are known in the art
(see WO 95/13399, Tyagi and Kramer, *ibid*). A broad
range of fluorophores in many different colours made
be used, including for example
35 5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid
(EDANS), fluorescein, FAM and Texas Red (see Tyagi,
Bratu and Kramer, 1998, *Nature Biotechnology*, 16, 49-

53. The use of probes labelled with different coloured fluorophores enables "multiplex" detection of two or more different probes in a single reaction vessel. A preferred quencher is

5 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), a non-fluorescent chromophore, which serves as a "universal" quencher for a wide range of fluorophores. The fluoescer and quencher moieties may be covalently attached to the probe in either orientation, either

10 with the fluoescer at or near the 5' end and the quencher at or near the 3' end or vice versa.

15 Protocols for the synthesis of molecular beacon probes are known in the art. A detailed protocol for synthesis is provided in a paper entitled "Molecular Beacons: Hybridization Probes for Detection of Nucleic Acids in Homogenous Solutions" by Sanjay Tyagi et al., Department of Molecular Genetics, Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA, which is available online via the PHRI website (at www.phri.nyu.edu or www.molecular-beacons.org)

25 Suitable combinations of the NASBA P1 and NASBA P2 primers may be used to drive a NASBA amplification reaction. In order to drive a NASBA amplification reaction the primer 1 and primer 2 oligonucleotides must be capable of priming synthesis of a double-stranded DNA from a target region of mRNA. For this to occur the primer 1 and primer 2

30 oligonucleotides must comprise target-specific sequences which are complementary to regions of the sense and the antisense strand of the target mRNA, respectively.

35 In the first phase of the NASBA amplification cycle, the so-called "non-cyclic" phase, the primer 1 oligonucleotide anneals to a complementary sequence in

the target mRNA and its 3' end is extended by the action of an RNA-dependent DNA polymerase (e.g. reverse transcriptase) to form a first-strand cDNA synthesis. The RNA strand of the resulting RNA:DNA hybrid is then digested, e.g. by the action of RNaseH, to leave a single stranded DNA. The primer 2 oligonucleotide anneals to a complementary sequence towards the 3' end of this single stranded DNA and its 3' end is extended (by the action of reverse transcriptase), forming a double stranded DNA. RNA polymerase is then able to transcribe multiple RNA copies from the now transcriptionally active promoter sequence within the double-stranded DNA. This RNA transcript, which is antisense to the original target mRNA, can act as a template for a further round of NASBA reactions, with primer 2 annealing to the RNA and priming synthesis of the first cDNA strand and primer 1 priming synthesis of the second cDNA strand. The general principles of the NASBA reaction are well known in the art (see Compton, J. Nature. 350: 91-92).

The target-specific probe oligonucleotides described herein may also be attached to a solid support, such as magnetic microbeads, and used as "capture probes" to immobilise the product of the NASBA amplification reaction (a single stranded RNA). The target-specific "molecular beacons" probes described herein may be used for real-time monitoring of the NASBA reaction.

Kits according to the invention may also including a positive control containing E6 and/or L1 mRNA from a known HPV type. Suitable controls include, for example, nucleic acid extracts prepared from cell lines infected with known HPV types (e.g. HeLa, CaSki).

Kits further may contain internal control amplification primers, e.g. primers specific for human U1A RNA.

- 5 Kits containing primers (and optionally probes) suitable for use in NASBA amplification may further comprise a mixture of enzymes required for the NASBA reaction, e.g. enzyme mixture containing an RNA directed DNA polymerase (e.g. a reverse
-
- 10 transcriptase), a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA (e.g. RNaseH) and an RNA polymerase. The RNA polymerase should be one which recognises the promoter sequence present in the 5'
- 15 terminal region of the NASBA P1 primers supplied in the reagent kit. The kit may also comprise a supply of NASBA buffer containing the ribonucleosides and deoxyribonucleosides required for RNA and DNA synthesis. The composition of a standard NASBA
- 20 reaction buffer will be well known to those skilled in the art (see also Leone et al., *ibid*).

Table 1: E6-specific sequences for inclusion in NASBA/PCR primers and probes

25

30

35

| SEQ ID | Primer /probe type | Sequence | HPV Type | nt |
|--------|--------------------|---------------------------------------|----------|-----|
| 1 | P2/PCR | CCACAGGAGCGACCCAGAAAGTTA | 16 | 116 |
| 2 | P1/PCR | X ₁ -ACGGTTTGTGTATTGCTGTTC | 16 | 368 |
| 3 | P2/PCR | CCACAGGAGCGACCCAGAAA | 16 | 116 |
| 4 | P1/PCR | X ₁ -GGTTTGTGTATTGCTGTTC | 16 | 368 |
| 5 | P1/PCR | X ₁ -ATCCCATCTCTATATACTA | 16 | 258 |
| 6 | P1/PCR | X ₁ -TCACGTGCGAGTAACTGT | 16 | 208 |
| 7 | P1/PCR | X ₁ -TTGCTTGCAGTACACACA | 16 | 191 |
| 8 | P1/PCR | X ₁ -TGCAGTACACACATTCTA | 16 | 186 |
| 9 | P1/PCR | X ₁ -GCAGTACACACATTCTAA | 16 | 185 |
| 10 | P2/PCR | ACAGTTATGCACAGAGCT | 16 | 142 |
| 11 | P2/PCR | ATATTAGAATGTGTGTAC | 16 | 182 |
| 12 | P2/PCR | TTAGAATGTGTGTACTGC | 16 | 185 |
| 13 | P2/PCR | GAATGTGTGTACTGCAAG | 16 | 188 |

| SEQ ID | Primer /probe type | Sequence | HPV Type | nt |
|--------|--------------------------|---|-------------|-----|
| 14 | PO | ACAGTTATGCACAGAGCT | 16 | 142 |
| 15 | PO | ATATTAGAATGTGTGTAC | 16 | 182 |
| 16 | PO | TTAGAATGTGTGTACTGC | 16 | 185 |
| 17 | PO | GAATGTGTGTACTGCAAG | 16 | 188 |
| 18 | PO | CTTTGCTTTTCGGGATTTATGC | 16 | 235 |
| 19 | PO | TATGACTTTGCTTTTCGGGA | 16 | 230 |
| 20 | MB | X ₂ -arm ₁ -TATGACTTTGCTTTTCGGGA-arm ₂ -X ₃ | 16 | 230 |
| 21 | P2/PCR | CAGAGGAGGAGGATGAAATAGTA | 16 | 656 |
| 22 | P1/PCR | X ₁ -GCACAACCGAAGCGTAGAGTCACAC | 16 | 741 |
| 23 | PO | TGGACAAGCAGAACCGGACAGAGC | 16 | 687 |
| 24 | P2/PCR | CAGAGGAGGAGGATGAAATAGA | 16 | 656 |
| 25 | P1/PCR | X ₁ -GCACAACCGAAGCGTAGAGTCA | 16 | 741 |
| 26 | PO | AGCAGAACCGGACAGAGCCCATTA | 16 | 693 |
| 27 | P2/PCR | ACGATGAAATAGATGGAGTT | 18 | 702 |
| 28 | P1/PCR | X ₁ -CACGGACACACAAAGGACAG | 18 | 869 |
| 28 | PO | AGCCGAACCACAACGTCACA | 18 | 748 |
| 30 | P2/PCR | GAAAACGATGAAATAGATGGAG | 18 | 698 |
| 31 | P1/PCR | X ₁ -ACACCACGGACACACAAAGGACAG | 18 | 869 |
| 32 | PO | GAACCACAACGTCACACAATG | 18 | 752 |
| 33 | MB | X ₂ -arm ₁ -GAACCACAACGTCACACAATG-arm ₂ -X ₃ | 18 | 752 |
| 34 | P2/PCR | TTCCGGTTGACCTTCTATGT | 18 | 651 |
| 35 | P1/PCR | X ₁ -GGTCGTCTGCTGAGCTTTCT | 18 | 817 |
| 36 | P2/PCR | GCAAGACATAGAAATAACCTG | 18 | 179 |
| 37 | P1/PCR | X ₁ -ACCCAGTGTTAGTTAGTT | 18 | 379 |
| 38 | PO | TGCAAGACAGTATTGGAAC | 18 | 207 |
| 39 | P2/PCR | GGAAATACCCTACGATGAAC | 31 | 164 |
| 40 | P1/PCR | X ₁ -GGACACAACGGTCTTTGACA | 31 | 423 |
| 41 | PO | ATAGGGACGACACACCACACGGAG | 31 | 268 |
| 42 | P2/PCR | GGAAATACCCTACGATGAACTA | 31 | 164 |
| 43 | P1/PCR | X ₁ -CTGGACACAACGGTCTTTGACA | 31 | 423 |
| 44 | PO | TAGGGACGACACACCACGGGA | 31 | 269 |
| 45 | P2/PCR | ACTGACCTCCACTGTTATGA | 31 | 617 |
| 46 | P1/PCR | X ₁ -TATCTACTTGTGTGCTCTGT | 31 | 766 |
| 47 | PO | GACAAGCAGAACCGGACACATC | 31 | 687 |
| 48 | P2/PCR | TGACCTCCACTGTTATGAGCAATT | 31 | 619 |
| 49 | P1/PCR | X ₁ -TGCGAATATCTACTTGTGTGCTCT GT | 31 | 766 |
| 50 | PO | GGACAAGCAGAACCGGACACATCCAA | 31 | 686 |
| 51 | MB | X ₂ -arm ₁ -GGACAAGCAGAACCGGACACATCCAA-arm ₂ -X ₃ | 31 | 686 |
| 52 | P2/PCR | ACTGACCTCCACTGTTAT | 31 | 617 |
| 53 | P1/PCR | X ₁ -CACGATTCCAAATGAGCCCAT | 31 | 809 |
| 54 | P2/PCR | TATCCTGAACCAACTGACCTAT | 33 | 618 |
| 55 | P1/PCR | X ₁ -TTGACACATAAACGAAGT | 33 | 763 |
| 56 | PO | CAGATGGACAAGCACAACC | 33 | 694 |
| 57 | P2/PCR | TCCTGAACCAACTGACCTAT | 33 | 620 |
| 58 | P1/PCR | X ₁ -CCCATAAGTAGTTGCTGTAT | 33 | 807 |
| 59 | PO | GGACAAGCACAACCGCCACAGC | 33 | 699 |
| 60 | MB | X ₂ -arm ₁ -GGACAAGCACAACCGCCACAGC-arm ₂ -X ₃ | 33 | 699 |

| SEQ ID | Primer /probe type | Sequence | HPV Type | nt |
|--------|--------------------------|---|-----------------|-----|
| 61 | P2/PCR | GACCTTTGTGTCTCAAGAA | 33 | 431 |
| 62 | P1/PCR | X ₁ -AGGTCAGTTGGTTCAGGATA | 33 | 618 |
| 63 | PO | AGAAACTGCACTGTGACGTGT | 33 | 543 |
| 64 | P2/PCR | ATTACAGCGGAGTGAGGTAT | 35 | 217 |
| 65 | P1/PCR | X ₁ -GTCTTTGCTTTTCAACTGGA | 35 | 442 |
| 66 | PO | ATAGAGAAGGCCAGCCATAT | 35 | 270 |
| 67 | P2/PCR | TCAGAGGAGGAGGAAGATACTA | 35 | 655 |
| 68 | P1/PCR | X ₁ -GATTATGCTCTCTGTGAACA | 35 | 844 |
| 69 | P2/PCR | CCCGAGGCAACTGACCTATA | 35 | 610 |
| 70 | P1/PCR | X ₁ -GTCAATGTGTGTGCTCTGTGA | 35 | 770 |
| 71 | PO | GACAAGCAAAACCAGACACCTCCAA | 35 | 692 |
| 72 | PO | GACAAGCAAAACCAGACACC | 35 | 692 |
| 73 | P2/PCR | TTGTGTGAGGTGCTGGAAGAAT | 52 | 144 |
| 74 | P1/PCR | X ₁ -CCCTCTCTTCTAATGTTT | 52 | 358 |
| 75 | PO | GTGCCTACGCTTTTTATCTA | 52 | 296 |
| 76 | P2/PCR | GTGCCTACGCTTTTTATCTA | 52 | 296 |
| 77 | P1/PCR | X ₁ -GGGGTCTCCAACACTCTGAACA | 52 | 507 |
| 78 | PO | TGCAAACAAGCGATTTCA | 52 | 461 |
| 79 | P2/PCR | TCAGGCGTTGGAGACATC | 58 | 157 |
| 80 | P1/PCR | X ₁ -AGCAATCGTAAGCACACT | 58 | 301 |
| 81 | P2/PCR | TCTGTGCATGAAATCGAA | 58 | 173 |
| 82 | P1/PCR | X ₁ -AGCACACTTACATACTG | 58 | 291 |
| 83 | PO | TGAAATGCGTTGAATGCA | 58 | 192 |
| 84 | PO | TTGCAGCGATCTGAGGTATATG | 58 | 218 |
| 85 | P2/PCR | TACACTGCTGGACAACAT | B(11) | 514 |
| 86 | P1/PCR | X ₁ -TCATCTTCTGAGCTGTCT | B(11) | 619 |
| 87 | P2/PCR | TACACTGCTGGACAACATGCA | B(11) | 514 |
| 88 | P1/PCR | X ₁ -GTCACATCCACAGCAACAGGTCA | B(11) | 693 |
| 89 | PO | GTAGGGTTACATTGCTATGA | B(11) | 590 |
| 90 | PO | GTAGGGTTACATTGCTATGAGC | B(11) | 590 |
| 91 | P2/PCR | TGACCTGTTGCTGTGGATGTGA | B(11) | 693 |
| 92 | P1/PCR | X ₁ -TACCTGAATCGTCCGCCAT | B(11) | 832 |
| 93 | PO | ATWGTGTGTCCCATCTGC | B(11) | 794 |
| 94 | P2/PCR | CATGCCATAAATGTATAGA | C(18) 39 45) | 295 |
| 95 | P1/PCR | X ₁ -CACCGCAGGCACCTTATTAA | C(18) 39 45 | 408 |
| 96 | PO | AGAATTAGAGAATTAAGA | C(18) 39 45 | 324 |
| 97 | P2/PCR | GCAGACGACCACTACAGCAAA | 39 | 210 |
| 98 | P1/PCR | X ₁ -ACACCGAGTCCGAGTAATA | 39 | 344 |
| 99 | PO | ATAGGGACGGGAACCACT | 39 | 273 |
| 100 | P2/PCR | TATTACTCGGACTCGGTGT | 39 | 344 |
| 101 | P1/PCR | X ₁ -CTTGGGTTTCTCTCTCGTGTTA | 39 | 558 |
| 102 | PO | GGACCACAAAACGGGAGGAC | 39 | 531 |
| 103 | P2/PCR | GAAATAGATGAACCCGACCA | 39 | 703 |
| 104 | P1/PCR | X ₁ -GCACACCACGGACACACAAA | 39 | 886 |
| 105 | PO | TAGCCAGACGGGATGAACACAGC | 39 | 749 |
| 106 | P2/PCR | AACCATTGAACCCAGCAGAAA | 45 | 430 |

| SEQ ID | Primer /probe type | Sequence | HPV Type | nt |
|--------|--------------------------|---|-----------------|-----|
| 107 | P1/PCR | X ₁ -TCTTTCTTGCCGTGCCTGGTCA | 45 | 527 |
| 108 | PO | GTACCGAGGGCAGTGTAAATA | 45 | 500 |
| 109 | P2/PCR | AACCATTTGAACCCAGCAGAAA | 45 | 430 |
| 110 | P1/PCR | X ₁ -TCTTTCTTGCCGTGCCTGGTCA | 45 | 527 |
| 111 | P2/PCR | GAAACCATTTGAACCCAGCAGAAA | 45 | 428 |
| 112 | P1/PCR | X ₁ -TTGCTATACTTGTGTTTCCCTACG | 45 | 558 |
| 113 | PO | GTACCGAGGGCAGTGTAAATA | 45 | 500 |
| 114 | PO | GGACAAACGAAGATTTTACA | 45 | 467 |
| 115 | P2/PCR | GTTGACCTGTTGTGTACCAGCAAT | 45 | 656 |
| 116 | P1/PCR | X ₁ -CACCACGGACACACAAAGGACAAG | 45 | 868 |
| 117 | P2/PCR | CTGTTGACCTGTTGTGTACGA | 45 | 654 |
| 118 | P1/PCR | X ₁ -CCACGGACACACAAAGGACAAG | 45 | 868 |
| 119 | P2/PCR | GTTGACCTGTTGTGTACGA | 45 | 656 |
| 120 | P1/PCR | X ₁ -ACGGACACACAAAGGACAAG | 45 | 868 |
| 121 | PO | GAGTCAGAGGAGGAAAACGATG | 45 | 686 |
| 122 | PO | AGGAAAACGATGAAGCAGATGGAGT | 45 | 696 |
| 123 | PO | ACAACCTACCAGCCCGACGAGCCGAA | 45 | 730 |
| 124 | P2/PCR | GGAGGAGGATGAAGTAGATA | 51 | 658 |
| 125 | P1/PCR | X ₁ -GCCCATTAACATCTGCTGTA | 51 | 807 |
| 126 | P2/PCR | AGAGGAGGAGGATGAAGTAGATA | 51 | 655 |
| 127 | P1/PCR | X ₁ -ACGGGCAAACAGGCTTAGT | 51 | 829 |
| 128 | PO | GCAGGTGTTCAAGTGTAGTA | 51 | 747 |
| 129 | PO | TGGCAGTGGAAAGCAGTGGAGACA | 51 | 771 |
| 130 | P2/PCR | TTGGGGTGCTGGAGACAAACATCT | 56 | 519 |
| 131 | P1/PCR | X ₁ -TTCATCCTCATCCTCATCCTCTGA | 56 | 665 |
| 132 | P2/PCR | TGGGGTGCTGGAGACAAACATC | 56 | 520 |
| 133 | P1/PCR | X ₁ -CATCCTCATCCTCATCCTCTGA | 56 | 665 |
| 134 | P2/PCR | TTGGGGTGCTGGAGACAAACAT | 56 | 519 |
| 135 | P1/PCR | X ₁ -CCACAACTTACACTCACAACA | 56 | 764 |
| 136 | PO | AAAGTACCAACGCTGCAAGACGT | 56 | 581 |
| 137 | PO | AGAACTAACACCTCAAACAGAAAT | 56 | 610 |
| 138 | PO | AGTACCAACGCTGCAAGACGTT | 56 | 583 |
| 139 | P1/PCR | X ₁ -TTGGACAGCTCAGAGGATGAGG | 56 | 656 |
| 140 | P2/PCR | GATTTTCCTTATGCAGTGTG | 56 | 279 |
| 141 | P1/PCR | X ₁ -GACATCTGTAGCACCTTATT | 56 | 410 |
| 142 | PO | GACTATTCACTGTATGGAGC | 56 | 348 |
| 143 | PO | CAACTGAYCTMYACTGTTATGA | A (16 31 35) | |
| 144 | MB | X ₂ -arm ₁ -CAACTGAYCTMYACTGTTATGA-arm ₂ -X ₃ | A (16 31 35) | |
| 145 | PO | GAAMCAACTGACCTAYWCTGCTAT | A (33 52 58) | |
| 146 | MB | X ₂ -arm ₁ -GAAMCAACTGACCTAYWCTGCTAT-arm ₂ -X ₃ | A (33 52 58) | |
| 147 | PO | AAGACATTATTCAGACTC | C (18 45 39) | |
| 148 | MB | X ₂ -arm ₁ -AAGACATTATTCAGACTC-arm ₂ -X ₃ | C (18 45 39) | |

Table 2: L1-specific sequences for inclusion in NASBA/PCR primers and probes

| | | | |
|----|--------|-------------------|--|
| 5 | SEQ ID | Primer/probe type | Sequence |
| | 149 | P2/PCR | AATGGCATTGTTGGGGTAA |
| | 150 | P1/PCR | X ₁ -TCATATTCCTCCCCATGTC |
| | 151 | PO | TTGTTACTGTTGTTGATACTAC |
| | 152 | P2/PCR | AATGGCATTGTTGGSRHAA |
| 10 | 153 | P1/PCR | X ₁ -TCATATTCCTCMMCATGDC |
| | 154 | PO | TTGTTACTGTTGTTGATACAC |
| | 155 | PO | TTGTTACTGTTGTTGATACCAC |
| | 156 | P2/PCR | AATGGCATTGTTGGSIHAA |
| | 157 | P2/PCR | AATGGCATTGTTGGIIHAA |
| 15 | 158 | P2/PCR | AATGGCATTGTTGGIRIAA |
| | 159 | P2/PCR | AATGGCATTGTTGGGGTAA |
| | 160 | P2/PCR | AATGGCATTGTTGGGGAAA |
| | 161 | P2/PCR | AATGGCATTGTTGGCATAA |
| | 162 | P2/PCR | AATGGCATTGTTGGGGCAA |
| 20 | 163 | P2/PCR | AATGGCATTGTTGGCACAA |
| | 164 | P1/PCR | X ₁ -TCATATTCCTCMICATGIC |
| | 165 | P1/PCR | X ₁ -TCATATTCCTCAACATGIC |
| | 166 | P1/PCR | X ₁ -TCATATTCCTCIICATGTC |
| | 167 | P1/PCR | X ₁ -TCATATTCCTCIICATGGC |
| 25 | 168 | P1/PCR | X ₁ -TCATATTCCTCIICATGAC 3' |
| | 169 | P1/PCR | X ₁ -TCATATTCCTCIICATGCC 3' |

Preferred primers suitable for use in detection of HPV L1 and E6 mRNA by NASBA are listed in the following tables. However, these are merely illustrative and it is not intended that the scope of

the invention should be limited to these specific molecules.

In the following Tables the NASBA P2 primers (p2) include the sequence GATGCAAGGTCGCATATGAG at the 5' end; the NASBA P1 primers (p1) include the sequence AATTCTAATACGACTCACTATAGGGAGAAGG at the 5' end. Oligonucleotides suitable for use as probes are identified by "po". The P2 primers generally contain HPV sequences from the postive strand, whereas the p1 primers generally contain HPV sequences from the negative strand. nt-refers to nucleotide position in the relevant HPV genomic sequence.

Table 3-Preferred E6 NASBA primers and probes

| Primer name | Sequence | HPV Type | nt |
|-------------|--|----------|-----|
| HAe6701p2 | GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAAGTTA | 16 | 116 |
| HAe6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGACGGTT TGTGTATTGCTGTTT | 16 | 368 |
| HAe6702p2 | GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAA | 16 | 116 |
| HAe6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGGTTTG TTGTATTGCTGTTT | 16 | 368 |
| HPV16p1 | AATTCTAATACGACTCACTATAGGGAGAAGGATTCCC ATCTCTATATACTA | 16 | 258 |
| HAe6702Ap1 | AATTCTAATACGACTCACTATAGGGAGAAGGTCA CGTCGCAGTAACTGT | 16 | 208 |
| HAe6702Bp1 | AATTCTAATACGACTCACTATAGGGAGAAGGTTG CTTGCACTACACACA | 16 | 191 |
| HAe6702Cp1 | AATTCTAATACGACTCACTATAGGGAGAAGGTGC AGTACACACATTCTA | 16 | 186 |
| HAe6702Dp1 | AATTCTAATACGACTCACTATAGGGAGAAGGGCA GTACACACATTCTAA | 16 | 185 |
| H16e6702Ap2 | GATGCAAGGTCGCATATGAGACAGTTATGCACAGAGCT | 16 | 142 |
| H16e6702Bp2 | GATGCAAGGTCGCATATGAGATATTAGAATGTGTGTAC | 16 | 182 |
| H16e6702Cp2 | GATGCAAGGTCGCATATGAGTATAGAAATGTGTGTACTGC | 16 | 185 |
| H16e6702Dp2 | GATGCAAGGTCGCATATGAGGAATGTGTGTACTGCAAG | 16 | 188 |
| H16e6702Apo | ACAGTTATGCACAGAGCT | 16 | 142 |
| H16e6702Bpo | ATATTAGAATGTGTGTAC | 16 | 182 |
| H16e6702Cpo | TTAGAATGTGTGTACTGC | 16 | 185 |

| | Primer name | Sequence | HPV Type | nt |
|----|-------------|---|----------|-----|
| | H16e6702Dpo | GAATGTGTGTACTGCAAG | 16 | 188 |
| | HAe6701po | CTTTGCTTTTCGGGATTTATGC | 16 | 235 |
| | HAe6702po | TATGACTTTGCTTTTCGGGA | 16 | 230 |
| 5 | HAe6702mb1 | X ₂ -cgcatgTATGACTTTGCTTTTCGGGAcatgcg -X ₃ | 16 | 230 |
| | HAe6702mb2 | X ₂ -ccagctTATGACTTTGCTTTTCGGGAagctgg -X ₃ | 16 | 230 |
| | HAe6702mb3 | X ₂ -cacgctTATGACTTTGCTTTTCGGGAgcgtg -X ₃ | 16 | 230 |
| 10 | | | | |
| | H16e6702mb4 | X ₂ -cgatcgTATGACTTTGCTTTTCGGGAcgatcg -X ₃ | 16 | 230 |
| | HAe6703p2 | GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGTA | 16 | 656 |
| | HAe6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCACAC | 16 | 741 |
| 15 | HAe6703po | TGGACAAGCAGAACCGGACAGAGC | 16 | 687 |
| | HAe6704p2 | GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGA | 16 | 656 |
| | HAe6704p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCA | 16 | 741 |
| | HAe6704po | AGCAGAACCGGACAGAGCCCATTA | 16 | 693 |
| | H18e6701p2 | GATGCAAGGTCGCATATGAGACGATGAAATAGATGGA GTT | 18 | 702 |
| | H18e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCACGGA CACACAAAGGACAG | 18 | 869 |
| 20 | H18e6701po | AGCCGAACCACAACGTCACA | 18 | 748 |
| | H18e6702p2 | GATGCAAGGTCGCATATGAGGAAAACGATGAAATAGA TGGAG | 18 | 698 |
| | H18e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGACACCA CGGACACACAAAGGACAG | 18 | 869 |
| | H18e6702po | GAACCACAACGTCACACAATG | 18 | 752 |
| 25 | H18e6702mb1 | X ₂ -cgcatgGAACCACAACGTCACACAATGcatgcg -X ₃ | 18 | 752 |
| | H18e6702mb2 | X ₂ -ccgtcgGAACCACAACGTCACACAATGcgacgg -X ₃ | 18 | 752 |
| | H18e6702mb3 | X ₂ -cggaccGAACCACAACGTCACACAATGggtccg -X ₃ | 18 | 752 |
| | H18e6702mb4 | X ₂ -cgatcgGAACCACAACGTCACACAATGcgatcg -X ₃ | 18 | 752 |
| | H18e6703p2 | GATGCAAGGTCGCATATGAGTTCCGGTTGACCTTCTA TGT | 18 | 651 |
| | H18e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGGTCGT CTGCTGAGCTTTCT | 18 | 817 |
| 30 | H18e6704p2 | GATGCAAGGTCGCATATGAGGCAAGACATAGAAATAA CCTG | 18 | 179 |
| | H18e6704p1 | AATTCTAATACGACTCACTATAGGGAGAAGGACCCAG TGTTAGTTAGTT | 18 | 379 |
| | H18e6704po | TGCAAGACAGTATTGGAAC | 18 | 207 |
| | H31e6701p2 | GATGCAAGGTCGCATATGAGGGAAATACCCTACGATG AAC | 31 | 164 |

| | Primer name | Sequence | HPV Type | nt |
|----|-------------|--|----------|-----|
| | H31e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGGACAC AACGGTCTTTGACA | 31 | 423 |
| | H31e6701po | ATAGGGACGACACACCACACGGAG | 31 | 268 |
| | H31e6702p2 | GATGCAAGGTTCGCATATGAGGGAAATACCCTACGATG AACTA | 31 | 164 |
| | H31e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCTGGAC ACAACGGTCTTTGACA | 31 | 423 |
| 5 | H31e6702po | TAGGGACGACACACCACACGGA | 31 | 269 |
| | H31e6703p2 | GATGCAAGGTTCGCATATGAGACTGACCTCCACTGTTA TGA | 31 | 617 |
| | H31e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTATCTA CTTGTGTGCTCTGT | 31 | 766 |
| | H31e6703po | GACAAGCAGAACCGGACACATC | 31 | 687 |
| | H31e6704p2 | GATGCAAGGTTCGCATATGAGTGACCTCCACTGTTATG AGCAATT | 31 | 619 |
| 10 | H31e6704p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTGCGAA TATCTACTTGTGTGCTCT GT | 31 | 766 |
| | H31e6704po | GGACAAGCAGAACCGGACACATCCAA | 31 | 686 |
| | H31e6704mb1 | X ₂ -ccgaaggGGACAAGCAGAACCGGACACATCC AAccttcgg -X ₃ | 31 | 686 |
| | H31e6704mb2 | X ₂ -ccgtcggGACAAGCAGAACCGGACACATCCA Acgacgg -X ₃ | 31 | 686 |
| | H31e6704mb3 | X ₂ -cacgtcggGACAAGCAGAACCGGACACATCCAA cgacgtg -X ₃ | 31 | 686 |
| 15 | H31e6704mb4 | X ₂ -cgcagcGGACAAGCAGAACCGGACACATCCAA gctgcg -X ₃ | 31 | 686 |
| | H31e6704mb5 | X ₂ -cgatcggGACAAGCAGAACCGGACACATCCAA cgatcg -X ₃ | 31 | 686 |
| | H31e6705p2 | GATGCAAGGTTCGCATATGAGACTGACCTCCACTGTTAT | 31 | 617 |
| | H31e6705p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCACGAT TCCAAATGAGCCCAT | 31 | 809 |
| | H33e6701p2 | GATGCAAGGTTCGCATATGAGTATCCTGAACCAACTGA CCTAT | 33 | 618 |
| 20 | H33e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTTGACA CATAAACGAACTG | 33 | 763 |
| | H33e6701po | CAGATGGACAAGCACAACC | 33 | 694 |
| | H33e6703p2 | GATGCAAGGTTCGCATATGAGTCCTGAACCAACTGACC TAT | 33 | 620 |
| | H33e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCCCAT AGTAGTTGCTGTAT | 33 | 807 |
| | H33e6703po | GGACAAGCACAACCAGCCACAGC | 33 | 699 |
| 25 | H33e6703mb1 | X ₂ -ccaagcGGACAAGCACAACCAGCCACAGCgct tgg -X ₃ | 33 | 699 |
| | H33e6703mb2 | X ₂ -ccaagcggGACAAGCACAACCAGCCACAGC cgcttgg -X ₃ | 33 | 699 |
| | H33e6703mb3 | X ₂ -cccagcGGACAAGCACAACCAGCCACAGCgct | 33 | 699 |

| | Primer name | Sequence | HPV Type | nt |
|----|-------------|---|----------|-----|
| | | ggg -X ₃ | | |
| | H33e6703mb4 | X ₂ -ccaaagcGGACAAGCACAACCAGCCACAGCg | 33 | 699 |
| | | cttttg -X ₃ | | |
| 30 | H33e6703mb5 | X ₂ -cctgcGGACAAGCACAACCAGCCACAGCgcagg -X ₃ | 33 | 699 |
| | H33e6703mb6 | X ₂ -cgatcgGGACAAGCACAACCAGCCACAGCcga | 33 | 699 |
| | | tcg -X ₃ | | |
| | H33e6702p2 | GATGCAAGGTCGCATATGAGGACCTTTGTGTCCTCAA GAA | 33 | 431 |
| | H33e6702p1 | AATTCGTAATAGGAGTCACTATAGGGAGAAGGAGGTCA GTTGGTTCAGGATA | 33 | 618 |
| | H33e6702po | AGAAACTGCACTGTGACGTGT | 33 | 543 |
| | H35e6701p2 | GATGCAAGGTCGCATATGAGATTACAGCGGAGTGAGG TAT | 35 | 217 |
| 35 | H35e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGTCTTT GCTTTTCAACTGGA | 35 | 442 |
| | H35e5601po | ATAGAGAAGGCCAGCCATAT | 35 | 270 |
| | H35e6702p2 | GATGCAAGGTCGCATATGAGTCAGAGGAGGAGGAAGA TACTA | 35 | 655 |
| | H35e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGATTAT GCTCTCTGTGAACA | 35 | 844 |
| | H35e6703p2 | GATGCAAGGTCGCATATGAGCCCGAGGCAACTGACCT ATA | 35 | 610 |
| 40 | H35e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGTCAAT GTGTGTGCTCTGTA | 35 | 770 |
| | H35e6702po | GACAAGCAAAACCAGACACCTCCAA | 35 | 692 |
| | H35e6703po | GACAAGCAAAACCAGACACC | 35 | 692 |
| | H52e6701p2 | GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTGGA AGAAT | 52 | 144 |
| | H52e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCCCTCT CTTCTAATGTTT | 52 | 358 |
| 45 | H52e6701po | GTGCCTACGCTTTTTATCTA | 52 | 296 |
| | H52e6702p2 | GATGCAAGGTCGCATATGAGGTGCCTACGCTTTTTAT CTA | 52 | 296 |
| | H52e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGGGGTC TCCAACACTCTGAACA | 52 | 507 |
| | H52e6702po | TGCAAAACAAGCGATTTC | 52 | 461 |
| 50 | H58e6701p2 | GATGCAAGGTCGCATATGAGTCAGGCGTTGGAGACATC | 58 | 157 |
| | H58e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGAGCAAT CGTAAGCACACT | 58 | 301 |
| | H58e6702p2 | GATGCAAGGTCGCATATGAGTCTGTGCATGAAATCGAA | 58 | 173 |
| | H58e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGAGCACA CTTACATACTG | 58 | 291 |
| | H58e6701po | TGAAATGCGTTGAATGCA | 58 | 192 |
| | H58e6702po | TTGCAGCGATCTGAGGTATATG | 58 | 218 |
| 55 | HBe6701p2 | GATGCAAGGTCGCATATGAGTACACTGCTGGACAACAT | B (11) | 514 |
| | HBe6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTCATCT | B (11) | 619 |

| | Primer name | Sequence | HPV Type | nt |
|----|-------------|--|-----------------|-----|
| | | TCTGAGCTGTCT | | |
| | HBe6702p2 | GATGCAAGGTCGCATATGAGTACACTGCTGGACAACA TGCA | B (11) | 514 |
| | HBe6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGTCACA TCCACAGCAACAGGTCA | B (11) | 693 |
| | HBe6701po | GTAGGGTTACATTGCTATGA | B (11) | 590 |
| 60 | HBe6702po | GTAGGGTTACATTGCTATGAGC | B (11) | 590 |
| | HBe6703p2 | GATGCAAGGTCGCATATGAGTGACCTGTTGCTGTGGA TGTGA | B (11) | 693 |
| | HBe6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTACCTG AATCGTCCGCCAT | B (11) | 832 |
| | HBe6703po | ATWGTGTGTCCCATCTGC | B (11) | 794 |
| | Hce6701p2 | GATGCAAGGTCGCATATGAGCATGCCATAAATGTATAGA | C (18 39 45) | 295 |
| 65 | Hce6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCACCGC AGGCACCTTATTAA | C (18 39 45) | 408 |
| | Hce6701po | AGAATTAGAGAATTAAGA | C (18 39 45) | 324 |
| | H39e6701p2 | GATGCAAGGTCGCATATGAGGCAGACGACCACTACAG CAAA | 39 | 210 |
| | H39e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGACACCG AGTCCGAGTAATA | 39 | 344 |
| | H39e6701po | ATAGGGACGGGGAACCACT | 39 | 273 |
| 70 | H39e6702p2 | GATGCAAGGTCGCATATGAGTATTACTCGGACTCGGTGT | 39 | 344 |
| | H39e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCTTGGG TTTCTCTTCGTGTTA | 39 | 558 |
| | H39e6702po | GGACCACAAAACGGGAGGAC | 39 | 531 |
| | H39e6703p2 | GATGCAAGGTCGCATATGAGGAAATAGATGAACCCGA CCA | 39 | 703 |
| | H39e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGCACAC CACGGACACACAAA | 39 | 886 |
| 75 | H39e6703po | TAGCCAGACGGGATGAACCACAGC | 39 | 749 |
| | HPV45p2 | GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA | 45 | 430 |
| | HPV45p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA | 45 | 527 |
| | HPV45po | GTACCGAGGGCAGTGTAATA | 45 | 500 |
| | H45e6701p2 | GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA | 45 | 430 |
| 80 | H45e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA | 45 | 527 |
| | H45e6702p2 | GATGCAAGGTCGCATATGAGGAAACCATTGAACCCAG CAGAAAA | 45 | 428 |
| | H45e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTTGCTA TACTTGTGTTCCCTACG | 45 | 558 |
| | H45e6701po | GTACCGAGGGCAGTGTAATA | 45 | 500 |
| | H45e6702po | GGACAAACGAAGATTTTCA | 45 | 467 |

| | Primer name | Sequence | HPV Type | nt |
|----|-------------|--|----------|-----|
| 5 | H45e6703p2 | GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CCAGCAAT | 45 | 656 |
| | H45e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCACCAC GGACACACAAAGGACAAG | 45 | 868 |
| | H45e6704p2 | GATGCAAGGTCGCATATGAGCTGTTGACCTGTTGTGT TACGA | 45 | 654 |
| | H45e6704p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCCACGG ACACACAAAGGACAAG | 45 | 868 |
| | H45e6705p2 | GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CGA | 45 | 656 |
| 10 | H45e6705p1 | AATTCTAATACGACTCACTATAGGGAGAAGGACGGAC ACACAAAGGACAAG | 45 | 868 |
| | H45e6703po | GAGTCAGAGGAGGAAAACGATG | 45 | 686 |
| | H45e6704po | AGGAAAACGATGAAGCAGATGGAGT | 45 | 696 |
| | H45e6705po | ACAACCTACCAGCCCCGACGAGCCGAA | 45 | 730 |
| | H51e6701p2 | GATGCAAGGTCGCATATGAGGGAGGAGGATGAAGTAG ATA | 51 | 658 |
| 15 | H51e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGCCCAT TAACATCTGCTGTA | 51 | 807 |
| | H51e6702p2 | GATGCAAGGTCGCATATGAGAGAGGAGGAGGATGAAG TAGATA | 51 | 655 |
| | H51e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGACGGGC AAACCAGGCTTAGT | 51 | 829 |
| | H51e6701po | GCAGGTGTTCAAGTGTAGTA | 51 | 747 |
| | H51e6702po | TGGCAGTGGAAGCAGTGGAGACA | 51 | 771 |
| 20 | H56e6701p2 | GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACA AACATCT | 56 | 519 |
| | H56e6701p1 | AATTCTAATACGACTCACTATAGGGAGAAGGTTTCATC CTCATCCTCATCCTCTGA | 56 | 665 |
| | H56e6702p2 | GATGCAAGGTCGCATATGAGTGGGGTGCTGGAGACAA ACATC | 56 | 520 |
| | H56e6702p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCATCCT CATCCTCATCCTCTGA | 56 | 665 |
| | H56e6703p2 | GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACA AACAT | 56 | 519 |
| 25 | H56e6703p1 | AATTCTAATACGACTCACTATAGGGAGAAGGCCACAA ACTTACACTCACAACA | 56 | 764 |
| | H56e6701po | AAAGTACCAACGCTGCAAGACGT | 56 | 581 |
| | H56e6702po | AGAACTAACACCTCAAACAGAAAT | 56 | 610 |
| | H56e6703po | AGTACCAACGCTGCAAGACGTT | 56 | 583 |
| | H56e6703po1 | TTGGACAGCTCAGAGGATGAGG | 56 | 656 |
| | H56e6704p2 | GATGCAAGGTCGCATATGAGGATTTTCCTTATGCAGT GTG | 56 | 279 |
| | H56e6704p1 | AATTCTAATACGACTCACTATAGGGAGAAGGGACATC TGTAGCACCTTATT | 56 | 410 |
| | H56e6704po | GACTATTTCAGTGTATGGAGC | 56 | 348 |
| | HPVAP01A | CAACTGAYCTMYACTGTTATGA | A (16 | |

| Primer name | Sequence | HPV Type | nt |
|-------------|--|-----------------|----|
| | | 31 35) | |
| HPVAp01Amb1 | X ₂ -cgcatgCAACTGAYCTMYACTGTTATGAcatgcg -X ₃ | A (16 31 35) | |
| HPVAp01Amb2 | X ₂ -ccgtcgCAACTGAYCTMYACTGTTATGAcga cgg -X ₃ | A (16 31 35) | |
| HPVAp01Amb3 | X ₂ -ccacccCAACTGAYCTMYACTGTTATGAgg gtgg -X ₃ | A (16 31 35) | |
| HPVAp01Amb4 | X ₂ -cgatcgCAACTGAYCTMYACTGTTATGAcga tcg -X ₃ | A (16 31 35) | |
| HPVAp04A | GAAMCAACTGACCTAYWCTGCTAT | A (33 52 58) | |
| HPVAp04Amb1 | X ₂ -ccaagcGAAMCAACTGACCTAYWCTGCTATgc ttgg -X ₃ | A (33 52 58) | |
| HPVAp04Amb2 | X ₂ -ccaagccGAAMCAACTGACCTAYWCTGCTAT ggcttgg -X ₃ | A (33 52 58) | |
| HPVAp04Amb3 | X ₂ -ccaagcgGAAMCAACTGACCTAYWCTGCTA Tcgcttgg -X ₃ | A (33 52 58) | |
| HPVAp04Amb4 | X ₂ -ccagcgGAAMCAACTGACCTAYWCTGCTATcg ctgg -X ₃ | A (33 52 58) | |
| HPVAp04Amb5 | X ₂ -cgatcgGAAMCAACTGACCTAYWCTGCTATcg atcg -X ₃ | A (33 52 58) | |
| HPVCp04 | AAGACATTATTCAGACTC | C (18 45 39) | |
| HPVCp04Amb1 | X ₂ -ccaagcAAGACATTATTCAGACTCgcttgg -X ₃ | C (18 45 39) | |
| HPVCp04Amb2 | X ₂ -cgcatgAAGACATTATTCAGACTCcatgcg -X ₃ | C (18 45 39) | |
| HPVCp04Amb3 | X ₂ -cccagcAAGACATTATTCAGACTCgctggg -X ₃ | C (18 45 39) | |
| HPVCp04Amb4 | X ₂ -cgatcgAAGACATTATTCAGACTCcgatcg -X ₃ | C (18 45 39) | |

Pairs of P1 and P2 primers having the same prefix (e.g. HAe6701p1 and HAe6701p2) are intended to be used in combination. However, other combinations may also be used, as summarised below for HPV types 16, 18, 31, 33 and 45.

Suitable primer-pairs for amplification of HPV 16 E6 mRNA are as follows:

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6701p1 or HAe6702p1 (both nt 368).

5 HAe6701p2 or HAe6702p2 (both nt 116) with HPV16p1 (nt 258).

H16e6702Ap2 (nt 142), H16e6702Bp2 (nt 182),
H16e6702Cp2 (nt 185) or H16e6702Dp2 (nt 188) with
HAe6701p1 or HAe6702p1 (both nt 368).

10

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6702Ap1 (nt 208), HAe6702Bp1 (nt 191), HAe6702Cp1 (nt 186) or HAe6702Dp1 (185). These combinations are suitable for amplification of all E6 splice variants.

15

HAe6703p2 or HAe6704p2 (both nt 656) with HAe6703p1 or HAe6704p1 (both nt 741). These combinations are suitable for amplification of all transcripts containing the E7 coding region (at least up to nt 741).

20

The following primer-pairs are preferred for amplification of HPV 18 E6 mRNA:

25 H18e6701p2 (nt 702) or H18e6702p2 (nt 698) with H18e6701p1 or H18e6702p1 (both nt 869).

H18e6703p2 (nt 651) with H18e6703p1 (nt 817).

30 H18e6704p2 (nt 179) with H18e6704p1 (nt 379).

The following primer-pairs are preferred for amplification of HPV 31 E6 mRNA:

H31e6701p2 or H31e6702p2 (both nt 164) with H31e6701p1 or H31e6702p1 (both nt 423).

5 H31e6703p2 (nt 617), H31e6704p2 (nt 619) or H31e6705p2 (nt 617) with H31e6703p1 (nt 766), H31e6704p1 (766) or H31e6705p1 (nt 809).

The following primer-pairs are preferred for amplification of HPV 33 E6 mRNA:

10

H33e6701p2 (nt 618) or H33e6703p2 (nt 620) with H33e6701p1 (nt 763) or H33e6703p1 (nt 807).

H33e6702p2 (nt 431) with H33e6702p1 (nt 618).

15

The following primer pair is preferred for amplification of HPV 45:

HPV45p2 (nt 430) with HPV45p1 (nt 527)

20

Table 4-E6 PCR primers

| | Primer name | Sequence | HPV type | nt |
|----|--------------|---------------------------|----------|-----|
| | HAe6701PCR2 | CCACAGGAGCGACCCAGAAAGTTA | 16 | 116 |
| 25 | HAe6701PCR1 | ACGGTTTGTGTATTGCTGTTC | 16 | 368 |
| | HAe6702PCR2 | CCACAGGAGCGACCCAGAAA | 16 | 116 |
| | HAe6702PCR1 | GGTTTGTGTATTGCTGTTC | 16 | 368 |
| | HAe6703PCR2 | CAGAGGAGGAGGATGAAATAGTA | 16 | 656 |
| | HAe6703PCR1 | GCACAACCGAAGCGTAGAGTCACAC | 16 | 741 |
| 30 | HAe6704PCR2 | CAGAGGAGGAGGATGAAATAGA | 16 | 656 |
| | HAe6704PCR1 | GCACAACCGAAGCGTAGAGTCA | 16 | 741 |
| | H18e6701PCR2 | ACGATGAAATAGATGGAGTT | 18 | 702 |
| | H18e6701PCR1 | CACGGACACACAAAGGACAG | 18 | 869 |
| | H18e6702PCR2 | GAAAACGATGAAATAGATGGAG | 18 | 698 |
| 35 | H18e6702PCR1 | ACACCACGGACACACAAAGGACAG | 18 | 869 |
| | H18e6703PCR2 | TTCCGGTTGACCTTCTATGT | 18 | 651 |
| | H18e6703PCR1 | GGTCGTCTGCTGAGCTTTCT | 18 | 817 |
| | H18e6704PCR2 | GCAAGACATAGAAATAACCTG | 18 | 179 |

| | Primer name | Sequence | HPV type | nt |
|----|--------------|-----------------------------|-----------------|-----|
| | H18e6704PCR1 | ACCCAGTGTTAGTTAGTT | 18 | 379 |
| | H31e6701PCR2 | GGAAATACCCTACGATGAAC | 31 | 164 |
| | H31e6701PCR1 | GGACACAACGGTCTTTGACA | 31 | 423 |
| | H31e6702PCR2 | GGAAATACCCTACGATGAAC | 31 | 164 |
| 5 | H31e6702PCR1 | CTGGACACAACGGTCTTTGACA | 31 | 423 |
| | H31e6703PCR2 | ACTGACCTCCACTGTTATGA | 31 | 617 |
| | H31e6703PCR1 | TATCTACTTGTGTGCTCTGT | 31 | 766 |
| | H31e6704PCR2 | TGACCTCCACTGTTATGAGCAATT | 31 | 619 |
| | H31e6704PCR1 | TGCGAATATCTACTTGTGTGCTCT GT | 31 | 766 |
| 10 | H31e6705PCR2 | ACTGACCTCCACTGTTAT | 31 | 617 |
| | H31e6705PCR1 | CACGATTCCAAATGAGCCCAT | 31 | 809 |
| | H33e6701PCR2 | TATCCTGAACCACTGACCTAT | 33 | 618 |
| | H33e6701PCR1 | TTGACACATAAACGAACG | 33 | 763 |
| | H33e6703PCR2 | TCCTGAACCACTGACCTAT | 33 | 620 |
| 15 | H33e6703PCR1 | CCCATAAGTAGTTGCTGTAT | 33 | 807 |
| | H33e6702PCR2 | GACCTTTGTGTCTCAAGAA | 33 | 431 |
| | H33e6702PCR1 | AGGTCAGTTGGTTCAGGATA | 33 | 618 |
| | H35e6701PCR2 | ATTACAGCGGAGTGAGGTAT | 35 | 217 |
| | H35e6701PCR1 | GTCTTTGCTTTTCAACTGGA | 35 | 442 |
| 20 | H35e6702PCR2 | TCAGAGGAGGAGGAAGATACTA | 35 | 655 |
| | H35e6702PCR1 | GATTATGCTCTCTGTGAACA | 35 | 844 |
| | H35e6703PCR2 | CCCGAGGCAACTGACCTATA | 35 | 610 |
| | H35e6703PCR1 | GTCAATGTGTGTGCTCTGTA | 35 | 770 |
| | H52e6701PCR2 | TTGTGTGAGGTGCTGGAAGAAT | 52 | 144 |
| 25 | H52e6701PCR1 | CCCTCTCTTCTAATGTTT | 52 | 358 |
| | H52e6702PCR2 | GTGCCTACGCTTTTTTATCTA | 52 | 296 |
| | H52e6702PCR1 | GGGGTCTCCAACACTCTGAACA | 52 | 507 |
| | H58e6701PCR2 | TCAGGCGTTGGAGACATC | 58 | 157 |
| | H58e6701PCR1 | AGCAATCGTAAGCACACT | 58 | 301 |
| 30 | H58e6702PCR2 | TCTGTGCATGAAATCGAA | 58 | 173 |
| | H58e6702PCR1 | AGCACACTTTACATACTG | 58 | 291 |
| | HBe6701PCR2 | TACACTGCTGGACAACAT | B(11) | 514 |
| | HBe6701PCR1 | TCATCTTCTGAGCTGTCT | B(11) | 619 |
| | HBe6702PCR2 | TACACTGCTGGACAACATGCA | B(11) | 514 |
| 35 | HBe6702PCR1 | GTCACATCCACAGCAACAGGTCA | B(11) | 693 |
| | HBe6703PCR2 | TGACCTGTTGCTGTGGATGTGA | B(11) | 693 |
| | HBe6703PCR1 | TACCTGAATCGTCCGCCAT | B(11) | 832 |
| | HCe6701PCR2 | CATGCCATAAATGTATAGA | C (18 39 45) | 295 |
| | HCe6701PCR1 | CACCGCAGGCACCTTATTAA | C (18 39 45) | 408 |
| 40 | H39e6701PCR2 | GCAGACGACCACTACAGCAAA | 39 | 210 |
| | H39e6701PCR1 | ACACCGAGTCCGAGTAATA | 39 | 344 |
| | H39e6702PCR2 | TATTACTCGGACTCGGTGT | 39 | 344 |
| | H39e6702PCR1 | CTTGGGTTTCTCTTCGTGTTA | 39 | 558 |
| | H39e6703PCR2 | GAAATAGATGAACCCGACCA | 39 | 703 |
| 45 | H39e6703PCR1 | GCACACCACGGACACACAAA | 39 | 886 |
| | H45e6701PCR2 | AACCATTGAACCCAGCAGAAA | 45 | 430 |
| | H45e6701PCR1 | TCTTTCTTGCCGTGCCTGGTCA | 45 | 527 |
| | H45e6702PCR2 | GAAACCATTGAACCCAGCAGAAAA | 45 | 428 |
| | H45e6702PCR1 | TTGCTATACTTGTGTTTCCCTACG | 45 | 558 |

| Primer name | Sequence | HPV type | nt |
|--------------|---------------------------|----------|-----|
| H45e6703PCR2 | GTTGACCTGTTGTGTTACCAGCAAT | 45 | 656 |
| H45e6703PCR1 | CACCACGGACACACAAAGGACAAG | 45 | 868 |
| H45e6704PCR2 | CTGTTGACCTGTTGTGTTACGA | 45 | 654 |
| H45e6704PCR1 | CCACGGACACACAAAGGACAAG | 45 | 868 |
| H45e6705PCR2 | GTTGACCTGTTGTGTTACGA | 45 | 656 |
| H45e6705PCR1 | ACGGACACACAAAGGACAAG | 45 | 868 |
| H51e6701PCR2 | GGAGGAGGATGAAGTAGATA | 51 | 658 |
| H51e6701PCR1 | GCCCATTAACATCTGCTGTA | 51 | 807 |
| H51e6702PCR2 | AGAGGAGGAGGATGAAGTAGATA | 51 | 655 |
| H51e6702PCR1 | ACGGGCAAACCAGGCTTAGT | 51 | 829 |
| H56e6701PCR2 | TTGGGGTGCTGGAGACAAACATCT | 56 | 519 |
| H56e6701PCR1 | TTCATCCTCATCCTCATCCTCTGA | 56 | 665 |
| H56e6702PCR2 | TGGGGTGCTGGAGACAAACATC | 56 | 520 |
| H56e6702PCR1 | CATCCTCATCCTCATCCTCTGA | 56 | 665 |
| H56e6703PCR2 | TTGGGGTGCTGGAGACAAACAT | 56 | 519 |
| H56e6703PCR1 | CCACAAACTTACACTCACAACA | 56 | 764 |
| H56e6704PCR2 | GATTTTCCTTATGCAGTGTG | 56 | 279 |
| H56e6704PCR1 | GACATCTGTAGCACCTTATT | 56 | 410 |

20

Preferred PCR primer-pairs for HPV types 16, 18, 31 and 33 are analogous to the NASBA primer-pairs.

25

Table 5-Preferred L1 NASBA primers and probes

30

35

| Primer name | Sequence |
|-------------|--|
| Onc2A2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGTAA 3' |
| Onc2A1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCCCATGTC 3' |
| Onc2PoA | 5' TTGTTACTGTTGTTGATACTAC 3' |
| Onc2B2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGSRHAA 3' |
| Onc2B1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMMCATGDC 3' |
| Onc2PoB | 5' TTGTTACTGTTGTTGATAC 3' |
| Onc2PoC | 5' TTGTTACTGTTGTTGATACCAC 3' |
| Onc2C2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGSIIAA 3' |
| Onc2D2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGIIHAA 3' |
| Onc2E2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGIRIAA 3' |
| Onc2F2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGTAA 3' |

| | | |
|----|--------|--|
| 5 | Onc2G2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGAAA 3' |
| | Onc2H2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGCATAA 3' |
| | Onc2I2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGCAA 3' |
| | Onc2J2 | 5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGCACAA 3' |
| | Onc2K1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMICATGIC 3' |
| 10 | Onc2L1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCAACATGIC 3' |
| | Onc2M1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGTC 3' |
| | Onc2N1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGGC 3' |
| | Onc2O1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGAC 3' |
| | Onc2P1 | 5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGCC 3' |

Table 6-Preferred L1 PCR primers

| | | |
|----|-------------|---------------------------|
| 15 | Primer name | Sequence |
| | Onc2A1-PCR | 5' AATGGCATTGTTGGGGTAA 3' |
| | Onc2A2-PCR | 5' TCATATTCCTCCCCATGTC 3' |
| | Onc2B1-PCR | 5' AATGGCATTGTTGGSRHAA 3' |
| | Onc2B2-PCR | 5' TCATATTCCTCMMCATGDC 3' |
| 20 | Onc2C1-PCR | 5' AATGGCATTGTTGGSIHAA 3' |
| | Onc2D1-PCR | 5' AATGGCATTGTTGGIIHAA 3' |
| | Onc2E1-PCR | 5' AATGGCATTGTTGGIRIAA 3' |
| | Onc2F1-PCR | 5' AATGGCATTGTTGGGGTAA 3' |
| | Onc2G1-PCR | 5' AATGGCATTGTTGGGGAAA 3' |
| 25 | Onc2H1-PCR | 5' AATGGCATTGTTGGCATAA 3' |
| | Onc2I1-PCR | 5' AATGGCATTGTTGGGGCAA 3' |
| | Onc2J1-PCR | 5' AATGGCATTGTTGGCACAA 3' |
| | Onc2K2-PCR | 5' TCATATTCCTCMICATGIC 3' |
| | Onc2L2-PCR | 5' TCATATTCCTCAACATGIC 3' |
| 30 | Onc2M2-PCR | 5' TCATATTCCTCIICATGTC 3' |
| | Onc2N2-PCR | 5' TCATATTCCTCIICATGGC 3' |
| | Onc2O2-PCR | 5' TCATATTCCTCIICATGAC 3' |
| | Onc2P2-PCR | 5' TCATATTCCTCIICATGCC 3' |

The HPV-specific sequences in SEQ ID NOs:149 and 150 (primers Onc2A2/Onc2A1-PCR and Onc2A1/Onc2A2-PCR) are identical to fragments of the HPV type 16 genomic sequence from position 6596-6615 (SEQ ID NO:149; 5 Onc2A2/Onc2A1-PCR), and from position 6729 to 6747 (SEQ ID NO:150; Onc2A1/Onc2A2-PCR).

The HPV-specific sequences SEQ ID NOs:152 and 153 (Onc2B2/Onc2B1-PCR and Onc2B1/Onc2B2-PCR) are variants 10 of the above sequences, respectively, including several degenerate bases. Representations of the sequences of degenerate oligonucleotide molecules provided herein use the standard IUB code for mixed base sites: N=G,A,T,C; V=G,A,C; B=G,T,C; H=A,T,C; 15 D=G,A,T; K=G,T; S=G,C;W=A,T; M=A,C; Y=C,T; R=A,G.

It is also possible to use variants of the HPV-specific sequences SEQ ID NO:152 (Onc2B2/Onc2B1-PCR) and SEQ ID NO:153 (Onc2B1/Onc2B2-PCR) wherein any two 20 of nucleotides "SRH" towards the 3' end of the sequence are replaced with inosine (I), as follows:

5' AATGGCATTGTGTTGGIIHAA 3'
5' AATGGCATTGTGTTGGSIIAA 3'
25 5' AATGGCATTGTGTTGGIRIAA 3'

The HPV-specific sequences SEQ ID NOs: 156-163 (present in primers Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2, Onc2J2, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, 30 Onc2I1-PCR and Onc2J1-PCR) are variants based on the HPV-specific sequence SEQ ID NO:152 (Onc2B2/Onc2B1-PCR), whereas the HPV-specific sequences SEQ ID NOs: 164-169 (present in primers Onc2K1, Onc2L1, Onc2M1, 35 Onc2N1, Onc2O1, Onc2P1, Onc2K2-PCR, Onc2L2-PCR,

Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR are variants based on the HPV-specific sequence SEQ ID NO:153 (Onc2B1/Onc2B2-PCR). These variants include degenerate bases and also inosine (I) residues. This sequence variation enables oligonucleotides incorporating the variant sequences to bind to multiple HPV types. Inosine bases do not interfere with hybridization and so may be included at sites of variation between HPV types in order to construct a "consensus" primer able to bind to multiple HPV types.

Any one or more of primers Onc2A2, Onc2B2, Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2 and Onc2J2, may be used in combination with any one or more of primers Onc2A1, Onc2B1, Onc2K1, Onc2L1, Onc2M1, Onc2N1, Onc2O1 and Onc2P1, for NASBA amplification of HPV L1 mRNA.

Any one or more of primers Onc2A1-PCR, Onc2B1-PCR, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR, may be used in combination with any one or more of primers Onc2A2-PCR, Onc2B2-PCR, Onc2K2-PCR, Onc2L2-PCR, Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR for PCR amplification of HPV L1 mRNA.

The invention will be further understood with reference to the following experimental examples:

Example 1-Detection of HPV mRNA by NASBA-based nucleic acid amplification and real-time detection

Collection and preparation of clinical samples

Pap smears and HPV samples were collected from 5970 women in the cervical screening program in Oslo,

Norway. Samples intended for RNA/DNA extraction were treated as follows:

5 Cervical samples were collected from each women
attending the cervical screening program using a
cytobrush (Rovers Medical Devices, The Netherlands).
The cytobrush was then immersed in 9 ml lysis buffer
(5M Guanidine thiocyanate). Since RNA is best
10 protected in the 5M guanidine thiocyanate at -70°C
only 1 ml of the total volume of sample was used for
each extraction round. The samples in lysis buffer
were stored at -20°C for no more than one week, then
at -70°C until isolation of DNA / RNA.

15 RNA and DNA were automatically isolated from 5300
women in the first round of extraction, using 1ml from
the total sample of 9ml in lysis buffer. RNA and DNA
were extracted according to the "Booms" isolation
method from Organon Teknika (Organon Teknika B.V.,
20 Boselind 15, P.O. Box 84, 5280 AB Baxtel, The
Netherlands; now Biomérieux, 69280 Marcy l'Etoile,
France) using the Nuclisens™ extractor following the
protocol for automated extraction.

25 Cell lines

DNA and RNA from HeLa (HPV 18), SiHa (HPV 16) and
CaSki (HPV 16) cell lines were used as positive
controls for the PCR and NASBA reactions. These cells
were also used as sample material in the sensitivity
30 study (Example 2). SiHa cells have integrated 1 - 2
copies of HPV 16 per cell, whilst CaSki cells have
between 60-600 copies of HPV 16, both integrated and
in the episomal state. HeLa cells have approximately
10-50 copies of HPV 18 per cell.

HPV detection and typing by PCR

Isolated DNA from cervical scrapes was subjected to PCR using the consensus GP5+/6+ primers (EP-B-0 517 704). The PCR was carried out in 50 µl reaction
5 volume containing 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 0.01 % Tween 20™, 200 mM each of dNTP, 1.5 mM MgCl₂, 1 U recombinant Taq DNA Polymerase (MBI Fermentas), 3 µl DNA sample and 50 pmol of each GP5+ and GP6+ primers. A 2 minutes denaturation step at
10 94°C was followed by 40 cycles of amplification with a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 1 minutes, a primer annealing step at 40°C for 2 minutes and a chain elongation step at 72°C for 1.5 minutes. The
15 final elongation step was prolonged by 4 minutes to ensure a complete extension of the amplified DNA.

The GP5+/6+ positive samples were subjected to HPV type 16, 31 and 33 PCR protocols as follows:
20 HPV 16, 31 and 33: The PCR was carried out in 50 µl containing 75 mM Tris-HCl (pH 8.8 at 25°C), 200 mM each of dNTP, 1.5 mM MgCl₂, 2.5 U recombinant Taq DNA Polymerase (MBI Fermentas), 3 µl DNA sample and 25 pmol of each primers. A 2 minutes denaturation step
25 at 94°C was followed by 35 cycles of amplification with a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 30 sec, a primer annealing step at 57°C for 30 sec and a chain elongation step at 72°C for 1 minutes. The
30 final elongation step was prolonged by 10 minutes to ensure a complete extension of the amplified DNA. The protocol for HPV 33 had a primer annealing step at 52°C. HPV 18 protocol: Primers were designed to identify HPV type 18. The PCR was carried out in 50 µl
35 containing 75 mM Tris-HCl (pH 8,8 at 25°C), 20 mM (NH₄)₂SO₄, 0.01 % Tween 20, 200 mM each of dNTP, 2.0 mM

MgCl₂, 2.5 U recombinant Taq DNA Polymerase (MBI Fermentas), 3 µl DNA sample and 25 pmol of each primer. A 2 minutes denaturation step at 94°C was followed by 35 cycles of amplification in a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 30 sec, a primer annealing step at 57°C for 30 sec and a chain elongation step at 72°C for 1 minutes. The final elongation step was prolonged by 10 minutes to ensure a complete extension of the amplified DNA.

A primer set directed against the human β-globin gene was used as a control of the DNA quality (Operating procedure, University Hospital Vrije Universiteit, Amsterdam, The Netherlands). The PCR was carried out in 50 µl containing 75 mM Tris-HCl (pH 8.8 at 25°C), 200 mM each of dNTP, 1,5 mM MgCl₂, 1 U Recombinant Taq DNA Polymerase (MBI Fermentas), 3 µl DNA sample and 25 pmol of each primer. A 2 minutes denaturation step at 94°C was followed by 35 cycles of amplification with a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 94°C for 1 minute, a primer annealing step at 55°C for 1 ½ minutes and a chain elongation step at 72°C for 2 minutes. The final elongation step was prolonged by 4 minutes to ensure a complete extension of the amplified DNA. HeLa was used as positive controls for HPV 18, while SiHa or CaSki were used as positive control for HPV 16. Water was used as negative control.

Primers used for HPV PCR:

| Type | Primer | Primer sequence | Position | Length (bp) |
|-------|--------|-----------------------------------|-----------|-------------|
| HPV16 | Pr1 | 5' TCA AAA GCC ACT GTG TCC TGA 3' | 421 - 440 | 119 |
| | Pr2 | 5' CGT GTT CTT GAT GAT CTG CAA 3' | 521 - 540 | |

| | | | | |
|--------|------|--|-------------|-----|
| HPV18 | Pr1 | (5' TTC CGG TTG ACC TTC TAT GT 3') | 651 - 670 | 186 |
| | Pr2 | (5' GGT CGT CTG CTG AGC TTT CT 3') | 817 - 836 | |
| HPV31 | Pr1 | 5' CTA CAG TAA GCA TTG TGC TAT GC 3' | 3835 - 3875 | 153 |
| | Pr2 | 5' ACG TAA TGG AGA GGT TGC AAT AAC CC 3' | 3963 - 3988 | |
| HPV33 | Pr1 | 5' AAC GCC ATG AGA GGA CAC AAG 3' | 567 - 587 | 211 |
| | Pr2 | 5' ACA CAT AAA CGA ACT GTG TGT 3' | 758 - 778 | |
| Gp+ | Gp5+ | 5' TTT GTT ACT GTG GTA GAT ACT AC 3' | 6624 - 6649 | 150 |
| | Gp6+ | 5' GAA AAA TAA ACT GTA AAT CAT ATT C | 6719 - 6746 | |
| BGPCO3 | Pr1 | 5' ACA CAA CTG TGT TCA CTA GC | | |
| BGPCO5 | Pr2 | 5' GAA ACC CAA GAG TCT TCT CT | | |

Visualization of the PCR products was done on a DNA 500 chip (Agilent Technologies, USA) according to their manual. The DNA chip uses micro scale gel electrophoresis with an optimal detection limit of 0.5 -50 ng/ml. The results were interpreted using the Bioanalyzer 2100 software (Agilent Technologies, USA).

NASBA RNA amplification

Precautions for avoiding contamination:

1. Perform nucleic acid release, isolation and amplification/detection in separate laboratory areas.
2. Store and prepare reagents for nucleic acid release, isolation and amplification/detection at the laboratory areas where nucleic acid release, isolation and amplification/detection are to be performed, respectively.
3. Keep all tubes and vials closed when not in use.
4. Pipettes and other equipment that have been used in one laboratory area must not be used in the other areas.
5. Use a fresh pipette or pipette tip for each pipetting action.
6. Use pipettes with aerosol resistant tips for fluids possibly containing nucleic acid. Pipetting of solutions must always be performed out of or into an

isolated tube that is opened and closed exclusively for this action. All other tubes and vials should be kept closed and separated from the one handled.

5 7. Use disposable gloves when working with clinical material possibly containing target-RNA or amplified material. If possible, change gloves after each pipetting step in the test procedure, especially after contact with possibly contaminated material.

10 8. Collect used disposable material in a container. Close and remove container after each test run.

9. Soak tube racks used during nucleic acid isolation or amplification/detection in a detergent (e.g. Merck Extran MA01 alkaline) for at least one hour after each test run.

15 The following procedure was carried out using reagents from the Nuclisens™ Basic Kit, supplied by Organon Teknika.

20 Procedure for n=10 samples:-

1. Prepare enzyme solution.

25 Add 55 µl of enzyme diluent (from Nuclisens™ Basic Kit; contains sorbitol in aqueous solution) to each of 3 lyophilized enzyme spheres (from Nuclisens™ Basic Kit; contains AMV-RT, RNase H, T7 RNA polymerase and BSA). Leave this enzyme solution at least for 20 minutes at room temperature. Gather the enzyme solutions in one tube, mix well by flicking the tube
30 with your finger, spin down briefly and use within 1 hour. Final concentrations in the enzyme mix are 375 mM sorbitol, 2.5 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase.

35 2. Prepare reagent sphere/KCl solution.

For 10 samples: add 80 μ l reagent sphere diluent (from Nuclisens™ Basic Kit; contains Tris/HCl (pH 8.5), 45% DMSO) to the lyophilized reagent sphere (from Nuclisens™ Basic Kit; contains nucleotides, dithiotreitol and $MgCl_2$) and immediately vortex well. Do this with 3 reagent spheres and mix the solutions in one tube.

10 ~~Add 3 μ l NASBA water (from Nuclisens™ Basic Kit) to the reconstituted reagent sphere solution and mix well.~~

15 Add 56 μ l of KCl stock solution (from Nuclisens™ Basic Kit) and mix well. Use of this KCl/water mixture will result in NASBA reactions with a final KCl concentration of 70 mM. Final concentrations in the reagent/KCl solution are 1 mM of each dNTP, 2 mM of ATP, UTP and CTP, 1.5 mM GTP, and 0.5 mM ITP, 0.5 mM dithiotreitol, 70 mM KCl, 12 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.5).

3. Prepare primer/probe solution containing target-specific primers and molecular beacon probe.

25 For each target reaction transfer 91 μ l of the reagent sphere/KCl solution (prepared in step 2) into a fresh tube. Add 25 μ l of primers/molecular beacon probe solution (to give final concentration of ~0.1-0.5 μ M each of the sense and antisense primers and ~ 15-70 pmol molecular beacon probe per reaction). Mix well

30 by vortexing. Do not centrifuge.

In case less than 10 target RNA amplifications are being performed refer to the table below for the appropriate amounts of reagent sphere solution, KCl/water solution and primers to be used. Primer

solutions should be used within 30 minutes after preparation.

| Reactions (n) | Reagent sphere solution (μl) | KCl/water (μl) | Primer mix (μl) |
|---------------|------------------------------|----------------|-----------------|
| 10 | 80 | 30 | 10 |
| 9 | 72 | 27 | 9 |
| 8 | 64 | 24 | 8 |
| 7 | 56 | 21 | 7 |
| 6 | 48 | 18 | 6 |
| 5 | 40 | 15 | 5 |
| 4 | 32 | 12 | 4 |
| 3 | 24 | 9 | 3 |
| 2 | 16 | 6 | 2 |
| 1 | 8 | 3 | 1 |

4. Addition of samples

For each target RNA reaction:

In a 96 well microtiter plate pipette 10 μl of the primer/probe solution (prepared in step 3) into each of 10 wells. Add 5 μl nucleic acid extract to each well. Incubate the microtiter plate for 4 minutes at 65 ± 1 °C. Cool to at 41 ± 0.5 °C for 4 minutes. Then to each well add 5 μl enzyme solution. Immediately place the microtiter plate in a fluorescent detection instrument (e.g. NucliSens™ EasyQ Analyzer) and start the amplification.

Results from clinical study

Table 7 shows the distribution of real-time NASBA HPV positive (L1 and/or E6 expression) and PCR HPV positive cases related to cytology results. PCR amplification was carried out as described by Karlsen et al., J Clin Microbiol. 34: 2095-2100, 1996. The figures for expected histology are based on average results from similar study on CIN III lesions (Clavel et al., Br J Cancer, 84: 1616-1623, 2001). The

results from several example cases are listed in Table 8.

Table 7:

| | | | | |
|-------------------------------|---------------|---------------|------------------|----------------|
| | Normal | Benign | Condyloma | CIN III |
| Cytology | 4474 | 66 | 16 | 15 |
| PCR | 9.0% | 44.6% | 87.5% | 73.3% |
| Real-time NASBA | 1% | 24.6% | 37.5% | 73.3% |
| Expected Histology | 0.2% | 5-15% | 15-20% | 71% |

Table 8:

| Internal No. | Cytology | PCR | L1 NASBA | E6 NASBA |
|---------------------|-----------------|------------|-----------------|-----------------|
| 84 | Neg | Neg | Neg | 31 |
| 289 | Neg | 31 | Pos | 31 |
| 926 | Neg | Neg | Pos | 16 |
| 743 | Benign | Neg | Neg | 33 |
| 1512 | Benign | 16 | Pos | 16 |
| 3437 | Benign | Neg | Neg | 18 |
| 3696 | Benign | 16 | Pos | Neg |
| 2043 | Condyloma | 16, 51 | Pos | 16 |
| 3873 | Condyloma | 16, 51 | Pos | 16 |
| 3634 | CIN II | 33 | Neg | 33 |
| 4276 | CIN III | Neg | Neg | 18 |
| 4767 | CIN III | 18 | Neg | 18 |
| 1482 | CIN III | Neg | Pos | 16 |
| 5217 | CIN III | 31 | Neg | 31 |
| 4696 | CIN III | Neg | Neg | Neg |

Example 2-Sensitivity of real-time NASBA on control cell lines

5 Cervical cancer cell lines, CaSki, SiHa and HeLa were diluted in lysis buffer either before automated extraction of nucleic acids using the Boom's extraction method from Organon Teknika/bioMerieux (parallels 1 and 3), or after nucleic acid extraction (parallel 2). Real-time NASBA was performed using molecular beacons probes labelled with Texas red (16, L1 and 18) or FAM (U1A, 33 and 31) following the protocol described above.

15 Table 9:

| Primer sets and probes | CaSki | | | | | | CaSki | | | | | | HeLa | | | | | |
|------------------------|-------|----|-------|----|-------|----|-------|-------|----|-------|----|-------|-------|-------|-------|-------|-------|-------|
| | 16 E6 | U1 | 16 E6 | U1 | 16 E6 | U1 | L1 | 33 E6 | L1 | 33 E6 | L1 | 33 E6 | 18 E6 | 31 E6 | 18 E6 | 31 E6 | 18 E6 | 31 E6 |
| 20 Parallels | 1 | 1 | 2 | 2 | 3 | 3 | 1 | 1 | 2 | 2 | 3 | 3 | 1 | 1 | 2 | 2 | 3 | 3 |
| Number of Cells | | | | | | | | | | | | | | | | | | |
| 100 000 | + | + | + | + | + | + | + | - | + | - | + | - | + | - | + | - | + | - |
| 10 000 | + | + | + | + | + | + | + | - | + | - | + | - | + | - | + | - | + | - |
| 25 1 000 | + | + | + | + | + | + | + | - | + | - | + | - | + | - | + | - | + | - |
| 100 | + | + | + | + | + | + | + | - | + | - | + | - | + | - | + | - | + | - |
| 10 | + | + | + | + | + | + | - | - | - | - | + | - | + | - | + | - | + | - |
| 1 | - | - | + | - | + | - | - | - | - | - | - | - | + | - | + | - | + | - |
| 30 10 ⁻¹ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Thus, it is possible to detect HPV E6 mRNA in less than 1 cell using real-time NASBA.

35 Real-time NASBA was tested both as a multiplex assay and as single reactions. The results from the following sensitivity study are based on parallel runs of CaSki, SiHa and HeLa cell lines, and on three parallel runs on synthetic DNA oligos for HPV type 16,

18, 31 and 33. The definition of the detection limit is that both of the samples in the parallel are positive. The number in the brackets (x) indicates that the specified amount of cells also have been
5 detected in some runs. Sensitivity is defined as the amount of cells necessary for detection of HPV in two parallel runs. The HPV types are determined from PCR and the specificity is based on NASBA compared to PCR.

10 Sensitivity

PCR: the HPV consensus PCR using Gp5+/6+ detected only down to 10^4 SiHa and HeLa cells, and down to 10^3 CaSki cells. However, the type specific PCR primer-sets were more sensitive, detecting 10^3 (10^2) SiHa cells and 0.1
15 CaSki cells for HPV 16 type specific PCR primer-set, while the HPV 18 type specific PCR primer-set detected 10^2 HeLa cells.

Real-time NASBA: Real-time NASBA with primers specific
20 for U1A, detected 10(1) SiHa and CaSki cells and 1 HeLa cell in the reaction mixture. For the HPV 16 specific primers, the lower detection limit was (10) (10^2 , 1) SiHa cells and 10 (1) CaSki cells and for the HPV 18 specific primers the detection limit was 1
25 (0.1) HeLa cell. The universal L1 primers detected 10 CaSki cells. HeLa cells and SiHa cells were not detected with the universal L1 primers.

Real-time multiplex NASBA with the U1A specific
30 primers, had a lower detection limit of 10^2 (10) SiHa cells and 10(1) CaSki cells when combined with the HPV 16 specific primers, which had a lower detection limit for 10(1) SiHa and 10(1) CaSki cells. The L1 specific primers in combination with the HPV 33 specific
35 primers detected 10^3 (102) CaSki cells. There was no

competing HPV 33 sample in the reaction. For the HPV 18 specific primers, the lower detection limit was 1 (0.1) HeLa cell when combined with the HPV 31 specific primers. There was no competing HPV 31 sample in the reaction. Sensitivity of the HPV 31 and HPV 33 specific primers were not tested, due to lack of cell lines harbouring these HPV types. They were tested against samples containing HPV 31 and HPV 33, but the amount of cells and the copy number of HPV 31 and HPV 33 in these cells were unknown and most probably varied in different samples.

Table 10: sensitivity of real-time NASBA compared to PCR

| Primer | NASBA | | | PCR | | |
|---------|----------------------|------------------------------------|---------|------------------------------------|-----------------|-----------------|
| | SiHa | CaSki | HeLa | SiHa | CaSki | HeLa |
| GP5+/6+ | - | - | - | 10 ⁴ | 10 ³ | 10 ³ |
| L1 | - | 10 ³ (10 ²) | - | - | - | - |
| U1A | 10 ² (10) | 10 (1) | - | - | - | - |
| HPV 16 | 10 (1) | 10 | - | 10 ³ (10 ²) | 0.1 | - |
| HPV 18 | - | - | 1 (0,1) | - | - | 10 ² |

Specificity: Cross reactivity of Real-time NASBA. Real-time NASBA primer combinations were tested against 78 cervical samples positive with PCR for HPV 6/11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 58 or HPV X to check for cross reactivity between HPV types. HPV X are samples positive for Gp5+/6+, but negative for the HPV types tested. 7 HPV 6/11, 14 HPV 16, 8 HPV 18, 7 HPV 31, 6 HPV 33, 9 HPV 35, 2 HPV 39, 4 HPV 45, 9 HPV 51, 1 HPV 52, 2 HPV 58 and 9 HPV X were tested and no cross reactivity was observed.

PCR: a total of 773 cervical samples were tested with PCR and the PreTect HPV-Proofer (Real time multiplex NASBA), and a total of 24.6% (190/773) samples were

positive with the Gp5+/6+ consensus PCR primers. 74.1% (83/112) were typed to be HPV 16, 13% (15/112) HPV 18, 17% (19/112) HPV 31 and 12% (13/112) HPV 33 including multiple HPV infections. A total of 103 samples had
5 single or multiple HPV infections, and 91.3% (94/103) had only a single HPV infection. Double HPV infections occurred in 8.7% (9/103) of the samples. All samples were first tested with the consensus Gp5+/6+ PCR primers. The HPV PCR negative samples from the
10 consensus Gp5+/6+ were then tested with β -globin control primers for a verification of intact DNA. The HPV PCR positive samples were not subjected to this DNA control. The HPV negative samples in this study were all positive with β -globin control PCR primers.
15 Only DNA samples positive with Gp5+/6+ PCR were subjected to HPV type specific PCR. HPV types of interest were HPV 16, 18, 31 and 33.

Real-time multiplex NASBA: For the real-time NASBA
20 reactions, the primers and probes for the U1A gene product were used as a performance control for intact RNA. Samples negative for U1A were rejected.
A total of 14.2% (110/773) of the samples were positive with at least one of the HPV type-specific
25 NASBA primers including samples showing multiple HPV infections. From these samples 54.5% (60/110) were positive with HPV 16 NASBA primers, 13.6% (15/110) with HPV 18 primers, 21.8% (24/110) with HPV 31 primers and 13.6% (15/110) with HPV 33 primers. A
30 total of 45 samples were positive with the L1 consensus primers and usually together with HPV 16 E6/E7 oncogene expression 82.2% (37/45). The consensus L1 was detected in 2.2% (1/45) together with either HPV 18, 31 and 33 respectively. L1 was also detected
35 alone in 8.9% (4/45) cases, and they all were PCR positive with Gp5+/6+ primers. A total of 108 samples

had single or multiple HPV infections, and 98.1% (106/108) had only a single HPV infections. Double mRNA expression occurred in 1.9% (2/108) of the samples.

5

Real-time multiplex NASBA compared to PCR: a total of 87 samples showed presence of HPV 16 DNA or RNA with HPV 16 PCR or PreTect HPV-Proofer. 64.4% (56/87) were determined to be positive for HPV 16 with both PCR and
10 real-time NASBA. 39.1% (34/87) were only positive with PCR and 3.4% (3/87) were positive only with real-time NASBA. For HPV 18, a total of 20 samples showed presence of HPV 18 DNA or RNA with either PCR or real-time NASBA. From these 20 samples, 50% (10/20) were
15 positive with both tests, and 35% (7/20) were only positive with PCR and 15% (3/20) were only positive with real-time NASBA. A total of 27 samples showed presence of HPV 31 DNA or RNA with either PCR or real-time NASBA. Out of these 27 samples, 59.3% (16/27)
20 were positive with both tests and 11.1% (3/27) were positive only with the PCR test and 18.5% (5/27) were only positive with the real-time NASBA test. For HPV 33, a total of 18 samples showed presence of HPV DNA or RNA with either PCR or PreTect-HPV Proofer and
25 55.6% (10/18) of the samples were tested positive with both tests. 16.7% (3/18) were only positive with PCR and 22.2% (4/18) were only positive with real-time NASBA.

30

Table 11: statistical distribution of HPV in samples with PCR and real-time NASBA

| | PCR | % | NASBA | % |
|-------------------------------|-----|------|-------|------|
| Total samples | 773 | | 773 | |
| Total positive samples | 190 | 24.6 | 110 | 14.2 |
| HPV16 | 83 | 74.1 | 60 | 54.5 |
| HPV 18 | 15 | 13 | 15 | 13.6 |
| HPV 31 | 19 | 17 | 24 | 21.8 |
| HPV33 | 13 | 12 | 15 | 13.6 |
| HPV X | 78 | 69.6 | - | - |

Table 12: correspondence between PCR and real-time NASBA

| | Total | Both tests | % | Only PCR + | % (PCR) | % (Total) | Only NASBA | % NASBA | % (total) |
|---------------|-------|------------|------|------------|---------|-----------|------------|---------|-----------|
| HPV 16 | 87 | 56 | 64.4 | 34 | 41.0 | 39.1 | 3 | 5 | 3.4 |
| HPV 18 | 20 | 10 | 50.0 | 7 | 46.7 | 35.0 | 3 | 20 | 15.0 |
| HPV 31 | 27 | 16 | 59.3 | 3 | 15.8 | 11.1 | 5 | 20.8 | 18.5 |
| HPV 33 | 18 | 10 | 55.6 | 3 | 23.1 | 16.7 | 4 | 26.7 | 22.2 |

Table 13: Real-time NASBA results for L1

| | Total | % |
|--------------------|-------|------|
| L1 (NASBA) | 45 | 100 |
| L1 + HPV 16 | 37 | 82.2 |
| L1 alone | 4 | 8.9 |
| L1 + HPV 18 | 1 | 2.2 |
| L1 + HPV 31 | 1 | 2.2 |
| L1 + HPV 33 | 1 | 2.2 |

Table 14: Specificity test of real-time NASBA compared to PCR

| HPV Type | 6/11 | 16 | 18 | 31 | 33 | 35 | 39 | 45 | 51 | 52 | 58 | X | Total |
|----------|------|----|----|----|----|----|----|----|----|----|----|---|-------|
| | 7 | 17 | 8 | 7 | 6 | 9 | 2 | 4 | 9 | 1 | 2 | 9 | 78 |
| 16 | - | 13 | - | - | - | - | - | - | - | - | - | - | - |
| 18 | - | - | 6 | - | - | - | - | - | - | - | - | - | - |
| 31 | - | - | - | 3 | - | - | - | - | - | - | - | - | - |
| 33 | - | - | - | - | 5 | - | - | - | - | - | - | - | - |

Discussion

Sensitivity of real-time NASBA was generally better than the sensitivity of PCR. The general sensitivity of real-time NASBA for all the markers were between 1 and 10^2 cells, which is considerable better than for the PCR reaction with a sensitivity range from 10^2 to 10^4 . As expected, the sensitivity of the specific primers and probes were better than the sensitivity of the universal primers and probes. Real-time NASBA was just as sensitive or more sensitive than real-time multiplex NASBA.

Real-time NASBA primers and molecular beacon probe directed towards U1A (a human house keeping gene) were used as a performance control of the sample material in the real-time NASBA reaction to ensure that the RNA in the sample material was intact. A positive signal from this reaction was necessary for a validation of the real-time NASBA reaction.

The sensitivity of the universal real-time NASBA with L1 (the major capsid protein of HPV) was much better than for the universal Gp5+/6+ PCR, also directed against L1, with a sensitivity of 10 cells compared to 10^3 (10^2) CaSki cells. These two primer sets (PCR and NASBA) have their targets in the same region of the conserved L1 gene of different HPV

types. The differences in sensitivity may be due to the fact that there is usually one copy of each gene per cell, while the copy number of mRNA may be several hundreds. The real-time NASBA L1 primers did not
5 detect SiHa or HeLa cells as the Gp5+/6+ PCR primers did, indicating lack of L1 expression in these cell lines. Gp5+/6+ PCR primers detected 10^4 SiHa or HeLa cells. Considering the amount of HPV copies in each cell, it makes sense that the CaSki cells were
10 detected in 1/10 the amount of cells from SiHa and HeLa since CaSki cells have 60-600 HPV copies per cell, both integrated and episomal, while SiHa cells have 1-2 HPV copies integrated per cell and HeLa cells have 10-50 HPV copies integrated per cell. The L1
15 primer set detected only CaSki cells, with both integrated and episomal forms of HPV, and not in SiHa or HeLa cells, with only integrated forms of HPV. This might indicate that the L1 gene is only expressed in episomal states of HPV infection, and therefore L1 may
20 be a valuable marker for integration and persistence of HPV infection.

The HPV type-specific NASBA primers are directed against the full length E6/E7 transcript, which are
25 expressed in large amount in cancer cells due to lack of E2 gene product. The real-time NASBA 16 type specific primers detected 10(1) SiHa cells and 10(1) CaSki cells compared to HPV 16 PCR primers that detected $10^3(10^2)$ SiHa cells. The explanation for this
30 might be the different amount of HPV copies in each cell line. The CaSki cells have both integrated and episomal forms of HPV, while SiHa has only integrated forms of HPV. This may be due to high expression of mRNA from the E6/E7 genes. For detection of CaSki
35 cells, the detection limit for the NASBA HPV 16 primers were 10(1) CaSki cells compared to 0.1 CaSki

cells for the HPV 16 PCR primers. This is peculiar,
but an explanation may be that the CaSki cells contain
from 60-600 copies of HPV 16 DNA, so that it is
possible to detect 0.1 CaSki cells with 6-60 HPV 16
5 DNA copies. The lower sensitivity of real-time NASBA
compared to PCR may indicate that the expression of
E6/E7 in the CaSki cells is moderate/low. Degradation
of the unstable mRNA may also be an explanation. The
amount of HPV copies in the CaSki cells may be in the
10 order of 60-600 times more than in the SiHa cells,
which is shown by the more sensitive detection of
CaSki cells.

The type specific HPV 18 PCR primers detected 10^2
15 HeLa cells. This is a magnitude of 100 better than the
HPV consensus Gp5+/6+ primers and states that specific
primers are generally more sensitive than consensus
primers. The sensitivity of the type specific HPV 18
NASBA primers was 1 (0,1) HeLa cells, indicating high
20 expression of E6/E7 in HeLa cells.

The sensitivity of U1A NASBA primers was 10 SiHa
or CaSki. The target for the U1A primer set is a human
housekeeping gene that is expressed in every human
25 cell.

The sensitivity of PCR and NASBA varies for
different primer sets and sample material, and
generally type specific primers are more sensitive
30 than consensus primers due to base pair mismatch in
consensus primer sets. The annealing temperature for
the primers in the PCR reaction can be optimised,
giving optimal reaction condition for the primers. In
contrast to the annealing temperature in PCR, the
35 annealing temperature for the NASBA primers must be
fixed at 41°C. This lack of temperature flexibility

may make the NASBA primers less sensitive and specific than the PCR primers.

5 PCR amplifies double stranded DNA and the target
is usually present as one copy per cell and this makes
it vulnerable to the number of cells in the sample
material. The target for the NASBA reaction is RNA,
and mRNA may be present as multiple copies per cell,
depending on the expression of the genes. By choosing
10 a gene that is highly expressed, the mRNA copy number
may be several hundred per cell and therefore easier
to detect.

15 dsDNA is relatively stable in the cell and the
material stays intact for a long time. In contrast to
dsDNA, mRNA is generally not very stable and
degradation of mRNA is rapid depending on the cell.
There is no detected DNase or RNase activity in the
lysis buffer so both dsDNA and ssRNA should be stable.
20 Autocatalytic activity may degrade both DNA and RNA.
The DNA/RNA from the cervix sample should stay intact,
when stored in the lysis buffer, for 24 hours at
15-30°C, 7 days at 2-8°C or at -70°C for long term
storage.

25

A limitation in the real-time NASBA reaction is
the concentration of the molecular beacon probes. The
amount of products will exceed the concentration of
the molecular beacon probes and therefore it will not
30 be detected because a high molecular beacon probe
concentration will make the reaction mixture more
complex and inhibit the amplification reaction.

Nucleotides may also be a limitation to the final
amount of the amplification product, both in the PCR
35 and in the NASBA reaction. The final concentration of

the amplified product may in itself inhibit further amplification because of the amount of product and the complexity of the reaction mixture. During a NASBA reaction in the presence of molecular beacons, the probe might compete with the amplification by hybridising to the template, making it unavailable for following RNA synthesis. In this way, RNA is subtracted as substrate for the reverse transcription steps and further RNA synthesis by T7 RNA polymerase. This competition is not significant with low amounts of molecular beacon, and with a high amount of molecular beacon this inhibition can be overcome by a higher number of copies of input RNA.

The linear relationship between the amount of input RNA and the time-to-positive signal was tested in a ten-fold serial dilutions of different HPV cell lines. There was a clear indication that a positive signal was dependent on the amount of input RNA and time. The multiplex reaction needed more time than the single reaction to show a positive signal. This might be due to competition in the more complex mixture in the multiplex reaction vessel and also to the fact that the multiplex reaction has a different and lower concentration of primer and probe. The relationship between amount of target RNA and time to positive signal opens up for a real-time multiplex quantitative amplification reaction with internal RNA standards in each reaction vessel.

Real-time NASBA: single vs. multiplex. Real-time NASBA was generally more sensitive than real-time multiplex NASBA. This was as expected because of competition between primers and probes in the multiplex reaction. The final concentration of primers and molecular beacon probes were optimised in the multiplex reaction

so that for at least one of the primer and probe sets the concentration were lower than in the single reaction. From this it follows that with a lower concentration of primers, the less sensitive the reaction, or at least the less rapid the reaction. It will take longer time to reach the exponential stage of the amplification reaction and therefore longer time to detect the products. The concentration of the

~~primers will not be a limitation to the final~~

concentration of the product in the NASBA reaction because the double stranded DNA created from the primers will continue to serve as a template for the RNA polymerase over and over again in a loop. The sensitivity of multiplex real-time NASBA was the same for HPV 16 and HPV 18 compared to single real-time NASBA, but the sensitivity for L1 decreased drastically from a detection limit of 10(1) in the single reaction to $10^3(10^2)$ CaSki cells in the multiplex reaction. For U1A NASBA primers, the sensitivity decreased from 10(1) to $10^2(10)$ SiHa cells, while the detection limit remained the same for the CaSki cells. This decrease in detection limit may be to more complex competition of primers and molecular beacon probes in the multiplex reaction. The final concentration of primers and molecular beacon probes may not be the best and the different primers and molecular beacon probes in the multiplex reaction may interfere with each other. The U1A NASBA primers detected 1 HeLa cell. One might expect the same detection level in all the cell lines, but the sensitivity of HeLa cells were 1/10 of the detection level of SiHa and CaSki cells. These cell lines are cancer cells and they might have different impact on the cells so that the expression of U1A is different. The differences may also be due to different amount of cells in each reaction, because of counting errors during harvesting of the cells.

Real-time NASBA showed no cross reactivity between HPV 16, 18, 31 and 33 or with HPV 6/11, 35, 39, 45, 51, 52, 58 or HPV X.

5 The specificity of the PCR reaction may be better than the specificity of the real-time NASBA reaction because the NASBA reaction is an isothermal reaction at 41°C with no possibilities to change the annealing
10 temperature of the primers. The primers are basically designed the same way as for the PCR primers. In a PCR reaction, you have the possibility to change the annealing temperature, in contrast to the NASBA reaction, and therefore choose an annealing
15 temperature that is optimal for the two primers. This makes the annealing of the primers more specific. The PCR results were visualized with gel electrophoresis. But the molecular beacon probes in the real-time NASBA reaction is an additional parameter compared to PCR and therefore may give the overall NASBA reaction a
20 better specificity. It is also easier to find two different regions on the DNA sequence for primer annealing because there is much greater flexibility in the length of the PCR product, than for the NASBA product, which should be less than 250 bp. It is
25 important for the specificity of the NASBA reaction to choose a unique area that is not conserved among the different HPV types. A couple of base pair mismatches may still give an amplification or hybridisation of the target.

30

 Detection of CaSki (integrated and episomal state) cells with the universal L1 NASBA primers and not SiHa or HeLa (both integrated) may give an indication that integrated HPV doesn't show any L1
35 expression, while HPV in the episomal state may have L1 expression.

In summary, an identification assay has been developed for HPV type 16, 18, 31 and 33 that can accurately identify the oncogenic E6/E7 expression of these HPV types. The assay can also identify the expression of the major capsid protein, L1.

Example 3-Further clinical study in 190 patients

10 Patients/Clinical samples

Biopsies from 190 women admitted to Østfold central-hospital for treatment of CIN in the period 1999-2001. The mean age of the 190 women included in the study was 37.4 years (range 22-74 years). Biopsies were frozen in -80°C immediately after collection.

Cytological examination of samples

The routine cytological reports were used to record cytological findings. No attempt was made to re-evaluate the slides. Each one of them indicated a CIN II-III condition, i.e. a high grade dysplasia or HSIL, which was the basis for hospital admittance, colposcopy and biopsy.

25 Histological examination of samples

A biopsy, here termed *biopsy 1*, was taken after a high-grade cytology report. If it confirmed a high-grade lesion (CIN II or III), the patient was again admitted to hospital, this time for colposcopically guided conization. Before the conization, but after local anesthesia was applied, a second biopsy (*biopsy 2*) was taken from an area of portio where a dysplasia was most likely to be localised, judged from the gross findings. This biopsy

(2 x 2 mm) was frozen within 2 minutes in a -80°C freezer.

Biopsy 2 was split in two when frozen and half was used for DNA/RNA extraction. The other half was fixed in 10% buffered formaldehyde and processed for histopathological examination. Some lesions were not correctly oriented in the paraffin block and had to be reoriented or serial sectioned in order to show the relevant surface epithelium. Consequently, it cannot be guaranteed that exactly the same tissue was used for the extraction and for the histopathological evaluation. The cone specimen, finally, was evaluated by the local pathologist, who in all cases could confirm the presence of dysplasia. It was not always the same grade as in the original biopsy, and, in many cases, not the same as in biopsy 2.

Extraction of nucleic acids

Nucleic acids were isolated using the automated Nuclisens Extractor as previously described (Boom et al., 1990). Each biopsy was cut in two pieces, one intended for histological examination and the other half for RNA analysis. The material intended for RNA analysis was divided into smaller pieces while kept on dry ice (-80°C) and put into 1 ml of lysisbuffer (as above) followed by 20 seconds of homogenisation using disposable pestles. 100 µl of the sample was further diluted 10 fold in lysisbuffer and 100 µl was then extracted for DNA/RNA. The extracted DNA/RNA was eluted with ~40 µl of elution buffer (Organon Teknika) and stored at -70°C.

All molecular beacon probes used in this study employ the fluorophore FAM (6-carboxyfluorescein) at

the 5'end of the structure. This was bound to a variable stem-loop sequence coupled to the universal quencher 4-(4'dimethylaminophenylazo)benzoic acid (DABCYL) at the 3'end. The probes were delivered by Eurogentec, Belgium. Final concentration of MBs used in the reaction was 2.5 mM. For the real-time NASBA we made use of the NucliSens Basic Kit (Organon Teknika, Netherlands), intended for the development of user-defined RNA amplification assays. The NASBA

amplification was carried out in a volume of 20 µl. The primer-sets and probes were directed against full-length E6/E7 mRNA for the high-risk HPV 16,18, 31, and 33. As performance control, to avoid false negative results due to degradation of nucleic acid, we used a primer set and probe directed against the human U1 small nuclear ribonucleoprotein (snRNP) specific A protein (U1A mRNA) (Nelissen et al., 1991). All samples were run in duplicate on separate machines (microplate readers for measuring fluorescence and absorbance, Bio-tek FL-600 FA from MWG). mRNA isolated from CaSki/SiHa or HeLa cells served as positive controls for HPV 16 and HPV 18 transcripts, respectively. Negative controls, included for every 7 reaction, consisted of a reaction containing all reagents except mRNA.

HPV DNA analysis; Polymerase Chain Reaction

The same extracts and amounts as used in the NASBA reaction were used for PCR. The L1 consensus primers Gp5+/Gp6+ were used to detect all samples containing HPV DNA. The PCR amplification was carried out as described above. The first DNA denaturation was done for 2 minutes at 94°C, then 40 cycles of PCR were run: denaturation 1 minute at 94°C, annealing for 2 minutes at 40°C, extension for 1.5 minutes at 72°C, followed by a final extension for 4 minutes at 72°C.

Typing of HPV was performed by using PCR type-specific primers against HPV 16, 18, 31, and 33 (6/11, 35, 45, 51, 52, 58), as described above.

5 Results

Originally 190 patients were biopsied after being given the diagnosis CIN I, CINII, or CIN III by cytology. A high-grade lesion was confirmed by histologically examination, 150 samples diagnosed as CIN III (78.9%). *Biopsy 2*, taken before conization, was used for RNA analysis. However, histological examination of this biopsy diagnosed only 53 samples of the originally 150 as CIN III [54 were given no diagnosis, 24 diagnosed as CIN II, 18 as CIN I, and 4 as HPV/condylom]. The number of CIN II samples increased from 16 (8,4%) to 30 (15,8%) [by Histology I 24 diagnosed as CIN III, 4 as CIN II, 1 as carcinom, and 1 as CIN I. 12 CIN II cases from Histology I were given a lower diagnosis in Histology II]. The degree of CIN I increased from 6 samples (3.2%) to 32 samples (16.8%). The 2 squamous cell carcinomas were in Histology II diagnosed as CIN III, the adenocarcinom as CIN II. In 71 samples (38.4%) high-grade lesions were not detected.

25

HPV oncogenic RNA was detected in 69 (36%) of the 190 patients. Of the 53 samples (28%) diagnosed as CIN III in Histology II, we found 40 (76%) cases showing HPV 16, 18, 31, or 33 oncogenic expression. In addition, we found oncogenic expression in 9 of 30 cases (30%) of CIN II, in 4 of 32 cases (13%) of CIN I, in 14 of 71 cases (20%) not showing cell abnormalities, and in 2 of 4 (50%) samples diagnosed as HPV/condyloma.

35

HPV 16 RNA was found in 42 of the 190 patients, HPV 18 was found in 7 (3.7%), HPV 31 in 15 (7.9%), and HPV 33 in 8 (4.2%). One patient had mixed infection with HPV 16 and HPV 18, and one with HPV 16 and HPV 31.

Using the consensus Gp5+/Gp6+ primers directed against the L1 gene, encoding the major capsid

protein, PCR detected HPV in 81 of the 190 cervical biopsies (43%). Of the 119 cases given a diagnosis in the second histological examination (115 diagnosed as CIN, 4 as HPV/condyloma) 63 were found to contain HPV DNA. The additional 18 cases detected were not given any histological diagnosis. 20 of the 81 cases were not detected by NASBA; 7 out of these were given the diagnosis CIN III, 2 were diagnosed as CIN II, 4 diagnosed as CIN I, and 7 given no diagnosis.

Type-specific PCR detected 85 cases containing HPV; 66 having HPV 16, 10 HPV 18, 14 HPV 31, 7 HPV 33. 12 cases had multiple infection: 3 with HPV 16+18; 4 with HPV 16+33, 5 with HPV 16+31. 20 no diagnosis.

Claims:

1. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or E6 mRNA are scored as being at risk of developing cervical carcinoma.

2. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of human papillomavirus (HPV) and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA;

Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

3. A method according to claim 1 which further
5 comprises screening for expression of p16^{ink4a}, wherein
subjects positive for expression of L1 and/or E6 mRNA
and positive for expression of p16^{ink4a} are scored as
being at risk of developing cervical carcinoma.

10 4. An *in vitro* method of screening human
subjects for the presence of integrated HPV or a
modified episomal HPV genome, which method comprises
screening the subject for expression of mRNA
15 transcripts from the L1 gene and the E6 gene of human
papillomavirus, wherein subjects negative for
expression of L1 mRNA but positive for expression of
E6 mRNA are scored as carrying integrated HPV or a
modified episomal HPV genome.

20 5. An *in vitro* method of screening human
subjects for the presence of integrated HPV or a
modified episomal HPV genome, which method comprises
screening the subject for expression of mRNA
25 transcripts from the E6 gene of human papillomavirus,
wherein subjects positive for expression of E6 mRNA
are scored as carrying integrated HPV or a modified
episomal HPV genome.

30 6. An *in vitro* method of screening human
subjects to assess their risk of developing cervical
carcinoma, which method comprises screening the
subject for expression of mRNA transcripts of the E6
gene of HPV and sorting the subject into one of two
categories of risk for development of cervical
35 carcinoma based on expression of E6 mRNA, wherein

individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified as high risk for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV or a modified episomal HPV genome and are therefore classified as no detectable risk for development of cervical carcinoma.

10

7. An *in vitro* method of identifying human subjects having abnormal cell changes in the cervix, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV, wherein individuals positive for expression of E6 mRNA are identified as having abnormal cell changes in the cervix.

8. A method according to any one of claims 5 to 7 wherein the human subjects are subjects previously identified as infected with human papillomavirus DNA in cells of the cervix.

9. A method according to any one of claims 5 to 7 wherein the human subjects are subjects having a previous diagnosis ASCUS, CIN 1 lesions or condyloma.

10. A method according to any one of claims 4 to 9 wherein individuals positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33 or 45 are scored as carrying integrated HPV.

11. A method according to any one of claims 1 to 4 which comprises screening for L1 mRNA expression

using a technique which is able to detect L1 mRNA from substantially all known HPV types.

5 12. A method according to any one of claims 1 to 11 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from at least one cancer-associated HPV type,

10 13. A method according to claim 12 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31, 33, and preferably 45.

15 14. A method according to any one of claims 1 to 13 wherein screening for L1 and/or E6 mRNA expression is carried using an amplification reaction to amplify of a region of the mRNA, together with real-time detection of the products of the amplification reaction.

20

15 15. A method according to claim 14 wherein screening for L1 and/or E6 mRNA expression is carried using real-time NASBA.

25 16. A kit for use in the detection of mRNA transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31, 33, and preferably 45 and
30 one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31, 33, and preferably 45.

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